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PhD Dissertation

Two-generation Reproductive Toxicity and Teratogenicity of 70% Ethanol Extract of *Moringa stenopetala* (Baker f.) Cufod. Leaf in Rats

By: Hussen Abdu Muhidin

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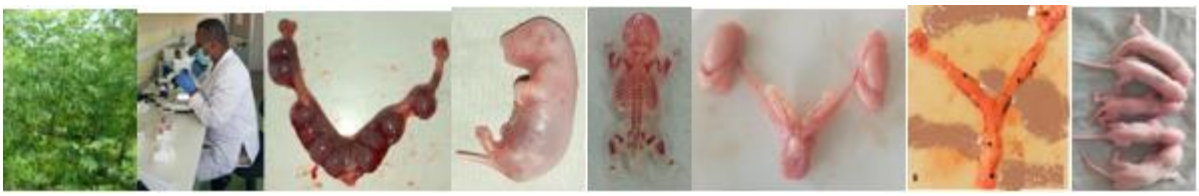

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By: Hussen Abdu Muhidin

A dissertation submitted to the School of Graduate Studies of Addis Ababa University, College of Health Sciences, School of Medicine, Department of Anatomy in Partial fulfillment of the requirements of the degree of Doctor of Philosophy (PhD) in Medical Anatomy

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Approval by the examining board

This is to certify that the Dissertation titled '**Two-generation reproductive toxicity and teratogenicity of 70% ethanol extract of *Moringa stenopetala* leaf in Rats**' is prepared by Hussen Abdu Muhidin, and submitted in partial fulfilment of the requirements for Doctor of Philosophy (PhD) in Medical Anatomy, and complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Chair of Department or Graduate Program Coordinator

AUTHOR'S DECLARATION

I firmly declared about the thesis titled **"Two-generation reproductive toxicity and teratogenicity of 70% ethanol extract of *Moringa stenopetala* leaf in Rats"**. The PhD dissertation presented here was carried out by me, Hussen Abdu Muhidin, in the Department of Anatomy, College of Health Sciences, Addis Ababa University in conjunction with TMMRD of EPHI from December 2018 to September 2021, under the direction and supervision of Prof. Girma Seyoum (Full Professor of Anatomy in the Department of Anatomy, School of Medicine, College of Health Sciences, Addis Ababa University). The dissertation is presented to the Department of Anatomy, College of Health Sciences, School of Graduate Studies, Addis Ababa University in order to partially meet the necessities for the Doctor of Philosophy (PhD) in Medical Anatomy.

This dissertation conforms with University rules and upholds the requirements for originality and quality. Any idea that was derived from a piece of literature has been acknowledged and referenced. The dissertation has never been submitted to another university for consideration of a degree or certificate.

Hussen Abdu Muhidin _____ _____

Name **Signature** **Date**

DEDICATION

It was not simple for a man to earn a Ph.D. after dropping out of school at the primary level for nine years to work in farming. It implies that I have a terrific person standing behind me. This doctoral dissertation is therefore dedicated to people who supported me throughout my entire wretched existence while I dropped out of school and helped me develop into a resilient individual in all circumstances. My parents, Mrs. Zehabu Mufti and Mr. Abdu Muhidin are my heroes and they merit my attention.

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ABBREVIATIONS/ACRONYMS

AAU	Addis Ababa University
AAUMF	Addis Ababa University, Medical Faculty
AGD	Anogenital Distance
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANOVA	One-way analysis of variance
AQ	Aqueous
AST	Aspartate Transaminase
Cm	Centimetre
CAM	Complimentary or alternative medicine
CRL	Crown-rump length
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
EPHI	Ethiopian Public Health Institute
EtHO	Ethanol
F1	First-generation
F2	Second-generation
FSH	Follicle stimulating hormone
G	Gram
GD	gestation day
GGT	Gamma-Glutamyl Transpeptidase
H	hour
H&E	Hematoxylin and eosin
Kg	kilogram
Km	Kilometre
KOH	potassium hydroxide
LD	Lactate Dehydrogenase
LD50	Median lethal dose
LH	Luteinizing hormone

m	meter
mg	milligram
mm	millimetres
mM	Millimole
M	Moringa
^{o}C	Degree centigrade
OECD	Organization for Economic Cooperation and Development
F0	Parental
PBS	Phosphate buffered saline
PI	Principal Investigator
PND	Postnatal day
SEM	Standard deviation of the mean
SPSS	Statistical package for social science
TM	Traditional medicine
TMMRD	Traditional and Modern medicine Research Directorate
USA	United State of America
WHO	World Health Organization
µl	Microliter
%	Percent
µm	micrometre

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Background: The plant *Moringa stenopetala* (Baker f.) Cufod. (Moringaceae) is a fast-growing tropical plant that is consumed as a native vegetable in southwest Ethiopia. The leaves of the plant are used as the main food source and in folks medicine several ailments, including elevated cholesterol, diabetes, stomach pain, hypertension, malaria, and retained placenta removal. There is conflicting data available right now regarding safety and effectiveness of *Moringa stenopetala.* According to certain research, it stimulates follicular growth, ovulation, and spermatogenesis. However, other research reports its teratogenic and abortifacient effects, induction of embryonic resorption at late stages of pregnancy, damage to the seminiferous tubules and epididymis, and post-coital antifertility effects. These conflicting reports and a lack of sufficient information regarding the plant's teratogenicity and reproductive toxicity profiles led to the design of this experiment. It is therefore important investigating into how the *Moringa stenopetala* leaf affects fetal and embryonic development as well as reproduction. Consequently, the aim of the present study was to evaluate the toxic effects of *Moringa stenopetala* leaf extract on the developing rat embryos and fetuses. Furthermore, the study investigated the toxicity of the plant on the reproductive organs and serum biochemical profiles of F0 and F1 male and female Wistar albino rats, as well as the developmental status of their offspring (F1 and F2 pups).

Methods: Fresh leaves *Moringa stenopetala* were collected, dried under shade at room temperature, powdered, and then extracted using a 70% ethanol. Five groups of animals, each consisting of ten pregnant rats, for each of the day 12 and day 20 experiments were used for teratogenicity study. The first three groups received 250 mg/kg (low dose), 500 mg/kg (middle dose), and 1000 mg/kg (high dose) body weight of *Moringa stenopetala* leaf extracts, respectively. The fourth and fifth groups were assigned as a pair-fed and *ad libitum* control groups. The plant extract was administered from the pregnancy of day 6 through 12. On the pregnancy days 12 and 20, respectively, the implanted embryos and fetuses were recovered from the membrane. Delays in the development and growth retardation were evaluated in the embryos and fetuses. In addition, the fetuses were inspected for obvious exterior deformities as well as for visceral organ and skeletal defects. The placenta's gross and histological alterations were also assessed.

For the two-generation reproductive toxicity study, the animals were randomly assigned in to five groups, each containing twenty animals for F0 (for parents) and F1 (first generation) parental animals, per sex per group, were employed. The dosage was the same as that employed in the teratogenicity study. The animals were treated for 10 weeks of premating, 2 weeks of mating periods. Female rats were dosed for additional 3 weeks of gestation and 3 weeks of lactation periods.

The animals were mated overnight by placing unrelated male rat in to a cage containing one virgin (nulliparous) female rat. Food consumption and weight of the animals were recorded weekly. In addition, reproductive parameters, estrous cycle, and pregnancy outcomes were also assessed. The weight and histopathology of reproductive organs were also evaluated. Data about body weights, estrous cycle, reproductive parameters, pregnancy outcomes, absolute and relative organ weights, and histopathology of reproductive organs, as well as serum reproductive hormone levels and serum biochemical levels were analysed. Each F1 and F2 pup was checked each day for clinical symptoms and mortality and weighed on postnatal day 0, 4, 7, 14, and 21. Anogenital distance and markers for sexual and developmental maturity were also evaluated using one-way ANOVA, followed by Tukey' post hock test and Dunnett's test.

Results: compared to the pair-fed control group, maternal daily consumption and weight gain were reduced in the high dose treated animals during the treatment and posttreatment periods, but not statistically significant. In addition, embryonic and fetal resorptions were significantly increased in 1000 mg/kg treated animals. The number of embryos and fetuses were also significantly decreased in the high-dose treated animals. Furthermore, the number of somites, yolk sac diameter, crown-ramp length and morphological scores of the embryos were significantly decreased in the high dose treated animals. Developmental delays in the otic system, somite score, and yolk sac circulation were observed in the high dose treated animals. In 1000 mg/kg treated pregnant rats, the fetal and placental weights were significantly reduced. Furthermore, in the high dose treated animals, the forelimb phalanges were absent in 40.7% of the fetuses. Histopathologic examination of the placenta in the high dose treated animals showed trophoblast proliferation, hematoma in the labyrinthine and trophoblastic zones, capillary dilation, decidual necrosis, decidual cytolysis, and decidual apoptosis.

However, neither the treatment nor the control groups revealed any gross morphological abnormalities in the visceral organs or the external genitalia.

In this two-generation reproductive investigation, weekly food intake and weight of the animals in F0 and F1 parental animals were decreased in the high dose treated groups. Furthermore, longer and irregular estrous cycle was observed in both F0 and F1 parental rats, yet, none of these were statistically significant. The gestation index was also significantly decreased in the high dose treated F0 rats (88.2%). In addition, the abortifacient index was significantly increased in 1000 mg/kg treated F0 rats (11.8%) as compared to the low dose and pair-fed control groups. Although the number of implantations and the total number of live births were significantly reduced in the high dose treated F0 and F1 parental animals. The viability index of the F1 and F2 litters on PND 4 was significantly lower in the middle dose and high dose treated animals.

The sex hormones like testosterone, FSH, and LH levels increased in 1000 mg/kg body weight treated F0 and F1 male parental animals. In the current study, the sperm count and motility were slightly increased in the high dose treated animals. In addition, the percentage of abnormal sperm cells decreased in the high dose treated animals. But these findings were not statistically significant. Absolute and relative reproductive organ weights as well as their histology in both sexes did not show significant differences. However, findings from liver and kidney function test showed significant variations. The serum AST, ALP, ALT, HDL and creatinine levels were significantly raised in the high dose treated F0 and F1 parental animals. Moreover, the LDL, urea and glucose levels were significantly reduced in the treatment groups of both F0 and F1 parental animals.

Conclusion: Administration of *Moringa stenopetala* leaves to the pregnant rats at a higher dose was found to have toxic and teratogenic effects on the developing rat embryos and fetuses. The plant extract increased embryonic and fetal resorptions at a higher dose. In addition, it delayed development of otic system, yolk sac circulation, and somite scores of embryos in the high dose treated pregnant animals. It also decreased the CRL, fetal and placental weights and change placental histopathology at a higher dose. However, the plant extract may increase reproductive hormones in both F0 and F1 male parental rats. Therefore, it

is recommended to limit excess consumption of *Moringa stenopetala* leaves during pregnancy. However, feeding of *Moringa stenopetala* leaves could help in boosting male reproduction.

Keywords: Developmental retardation, Embryo, Fetus, Gestation, *Moringa stenopetala*, Placenta, Reproductive toxicity, Rat, Teratogenicity, Two-generation

CHAPTER ONE

1. INTRODUCTION

1.1.Background

1.1.1. Importance of Traditional Medicine

According to World Health Organization (WHO), traditional medicine (TM) is a health practices, methods, knowledges, and beliefs that include plants, mineral based medicines, animals, spiritual, manual techniques and physical exercises and are used exclusively or in combination to diagnose, treat, and prevent ailments and reservation of societal well-being [1]. The use of traditional medicine is currently growing in the developed countries and it is popular across the developing nations [2].

Furthermore, according to WHO estimates, over 80% of people in developing countries use TM as their primary method for receiving medical care. In addition, the use of complementary or alternative medicine (CAM), notably herbal medicines, has also seen a rise in developed countries, particularly over the past few decades [3, 4]. For instance, in China, traditional herbal treatments make about 30% to 50% of all medical usage [2, 5].

Traditional medicine, a significant socio-cultural legacy in Africa that has allegedly existed for several hundreds of years, was falsely portrayed as being hostile, especially by Western faiths that date back to the colonial era. Today, however, TM is focused on to achieve the objectives of expanding the delivery of basic healthcare. In African countries, about 80% of the people use traditional medicine as to their first alternative for their healthcare activities [6]. For instance, in Mali, Ghana, Zambia, and Nigerian population, herbal preparation at their home is their first alternative and first line therapy to manage fever brought on by malarial infection [2, 5].

1.1.2. Ethiopian Traditional Medicine

It is hard to pinpoint the moment when medicine was invented in Ethiopia, but there is no doubt that the development of therapeutic methods closely corresponds to the course of diseases. Most commonly methods used by Ethiopian traditional medical practitioners to treated different types of diseases are herbal remedies, spiritual healings, bone settings, and minor surgical operations. Ethiopian TM is also quite complicated, diversified, and varies widely across various ethnic groups. The majority of Ethiopian folk medicinal practices

utilize a holistic approach to therapy and depend on clarification of diseases that includes both "mystical" as well as "natural" origins of the disease [7]. This suggests that our country Ethiopia has a long history in the use of TM and has advanced strategies to manage it [8].

Due to the local pharmacopeia's cultural acceptance, the relative affordability of the traditional medicine, the challenges to access modern health care, up to 80% of the Ethiopian population utilize traditional medicines [2]. Various studies revealed that most of the rural residents of Ethiopia depend on the traditional healers for their health care services, with others spinning to traditional remedies when modern health care facilities fall short for a variety of reasons [9]. Frustration with contemporary medicine and its lack of effectiveness, particularly for the situations of various diseases like cancer, liver problems, herpes zoster, eczema, swelling, haemorrhoids were some of the reasons why the traditional medicines were preferred over the conventional treatments [10].

1.1.3. Traditional Medicinal Plants

Animals, humans, and the environment have all depended on plants throughout history and will continue to do so [11]. Meanwhile early eras, plants have been served as a good source of medicine [12]. Globally, more than 3.5 billion people depend on plant based medications to address both human and animal diseases [13]

It is known that herbal medicine is one of the eldest forms of therapy for a variety of diseases, and it takes benefited from a reasonably high subscription for apparent explanations like being affordable, and accessible, and it fits with the socio-cultural life of the population. According to the WHO, medicinal or herbal plants are the best possible way to obtain a variety of drugs. In developed nations like the United States, it is believed that plant-based medications contribute up to 25% of all drugs, but in developing nations like China and India, they can make up as much as 80% [14]. They generate primary and secondary metabolites with a diverse range of actions. The secondary metabolites, which include terpenoids, phytosterols, tannins, phenolic compounds, and alkaloids, are the most significant of these bioactive components of plants [15].

1.1.3.1.Ethiopian Traditional Medicinal Plants

In Ethiopia, plant-based ingredients make up more than 95% of TM preparations [16]. Ethiopian growers of medicinal plants utilize over 800 different species to address about 300 different medical conditions [17]. There are many different kinds of medicinal plants, like herbs, shrubs, trees, and climbers. Among these, herbal remedies are the most commonly employed medicinal plants in Ethiopia to cure both people and animals [18].

In Ethiopia, the same herbal components that are used in the traditional medicine are also used as ingredients and spices in Ethiopian food. However, these herbs and spices are used in very small amounts. Thus, consuming them as a part of normal diet is unlikely to cause harmful herb-drug interaction. However, there might be a higher probability of adverse interactions with conventional medicines when these herbs and spices are used for therapeutic purposes [19].

1.1.3.2. Evaluation of Safety Profile of Herbal Medicines

The use of traditional medicine is recognized in many developed countries across the globe. The application of complementary and alternative medicine has been very popular in North America, Europe, and Australia [20]. In these areas, people use herbal medicines as blood cleansers, diet supplements, and body-size reducers to encourage healthy lifestyles. This treatment approach significantly lowers the cost of purchasing pricey medications from the pharmacy, which supports the expansion of the market for herbal goods in these areas [21].

Despite the many benefits of folklore medicines, their safety for the general population is constantly being questioned [22]. Less than 10% of the herbal products available on the global market are really standardized to the recognized active ingredients, and stringent quality control procedures are not always strictly followed. Even in the absence of a scientific assessment to determine their safety, the marketing of herbal concoctions is a standard in African communities. In addition, the local community believed that products from herbs are harmless. Moreover, the absence of regulatory guidelines governing the quality and safety of the products offered by traditional healers has resulted in millions of people being duped and in some cases losing their lives [22, 23].

Numerous plants contain potentially toxic bioactive chemicals that might interfere with ordinary functional processes of the body and could chelate cellular constituents like proteins and the genetic material deoxyribonucleic acid (DNA), which leads to toxicity and mutation of the cells and their constituents [24]. Furthermore, it has been found that some plant species can indeed be cytotoxic in high dosages. This merely implies that a substance might become hazardous at a high dose while being highly safe in the low dosages [22].

The WHO recommends that prior to the incorporation of herbal products into the healthcare system, it is crucial to determine their safety. A key component of quality control is also safety evaluation [25]. It should be encouraged to use examinations that are challenging or else perhaps unbearable to notice clinically when evaluating the safety of medicinal plants. Therefore, it is recommended to evaluate immunotoxicity, genotoxicity, carcinogenicity, teratogenicity, and reproductive toxicity of the herbal products [26, 27].

1.1.3.3.Evaluation of Teratogenicity of Medicinal Plants

Teratogenic agents are substances that, when exposed to a fetus, can alter the fetus' morphology or future function. These substances can also be infectious agents, physical illnesses, or deficits. During times of rapid differentiation, embryos and fetuses are more sensitive and susceptible to teratogenic agents [28-30]. A maximum of 2–3% of birth abnormalities are caused by drug use. Almost any medication that the woman takes while she is pregnant has the potential to be harmful to the fetus and cause congenital malformations [31].

Teratogenicity is dependent on the teratogen's capacity to cross the placenta [28]. A large majority of lipid-soluble substances easily cross the placenta. When their molecular weight is reduced, water-soluble compounds may pass through placenta more readily. The amount of drugs that is free to cross the placental is influenced by the ability of the drug to bound with plasma proteins [31].

The ability to avoid exposure at crucial developmental stages and avoid some congenital deformities is made possible by the information of the teratogens. To reduce exposure of pregnant women to the teratogenic agents, it is important to evaluate and identify the teratogenicity of herbal products, drugs, food additives, and pesticides [28, 32].

1.1.3.4. Reproductive and Developmental Toxicity of Medicinal Plants

The intricate interconnections of various systems that affect all features of sexual behaviour, sexual function, spermatogenesis, ejaculation in males, and conception and parturition in females are what lead to normal reproductive capability in both men and women [33]. Moreover, exposure to certain xenobiotic-reproductive toxicants through occupational exposure, lifestyle choices, or chemotherapy may have an impact on reproductive functioning [34].

The term "reproductive toxicity" describes structural and functional alterations that have an impact on the ability of sexually mature males and females to reproduce [35, 36] and developmental toxicity in the offspring [36].

Reproductive toxicity in both males and females can lead to injury to the reproductive organs, changes in the way the hormones control gametogenesis and their release, abnormal copulating behaviour, changed sexual function, changes in the endocrine system, or a general decline in fertility. In female animals, reductions of fertility index, number of implantations, elapsed time between mating, or fecundity are frequently used to identify decreased fertility. Furthermore, alteration in the commencement of parturition, as well as atypical or challenging deliveries (dystocia), are all toxicities that have an impact on labor and delivery in female animals. Drugs may also change a nursing mother's lactation process (such as the quality or amount of milk produced), or they may change the mother's behaviour toward her nursing offspring [35].

Developmental toxicity describes deleterious consequences for the developing organism that follow exposure before conception, throughout pregnancy, or postnatally until sexual maturity. Developmental toxicity leads to four main consequences that includes; death, dysmorphogenesis (structural abnormalities), growth alterations, and functional impairment. Mortality related to developmental toxicity can happen at any moment, starting from early pregnancy through postweaning period. For instance, embryo-fetal death is a subdivision of death caused as of developmental toxicity. Developmental toxicity might therefore manifest as pre- or peri-implantation loss, early or late resorption, miscarriage, still birth, neonatal mortality, or peri-weaning loss [35].

Furthermore, the results of dysmorphogenesis are typically observed as structural anomalies, such as changes in the soft tissues or skeleton of the offspring. Growth

alterations are often categorized as early maturation, excessive growth, or growth retardation. The most common method for evaluating growth rate would be by monitoring body weight of the animals. In addition, the growth rate of the animals would be assessed by measuring the anogenital distance (AGD) and crown ramp length (CRL). Functional toxicities might be any long-lasting deviation from normal physiologic or biochemical functions, although often only reproductive function and developmental neurobehavioral impacts are evaluated. During developmental toxicity studies, assessment of locomotion, reflex development, sexual maturation, memory, mating behaviour, and fertility are commonly conducted [35].

Currently, herbal products are mostly used to manage many forms of problems of reproductive function. Yet, impurities like organic pollutants and heavy metals that trapped from the environment and absorb by medicinal plants, particularly when their absorptions are over the tolerable level, they interfere with the human reproductive system's normal physiological function [22, 33, 37]. According to certain *in vivo* investigations, the toxicity of contaminants related to medicinal plants caused decreases in the weight of sex organs, sexual performance, the rate of implantation, fertility parameters, as well as chromosomal aberration and mitotic inhibition [38, 39]. Increased concentrations of these pollutants have the potential to cause oxidative stress by causing the generation of free radicals that can cause oxidative damage to tissues, organs, and macromolecules and impair reproductive systems [40, 41]. According to Nwangwa's findings, for instance, *Xylopia aethiopica* ethanolic extract displayed a decrease in the sperm motility, sperm viability, and sperm count in rats [42]. In another study, *Andrographis paniculata* reduced spermatogenesis and triggered degenerative alterations in the rat seminiferous tubules [43]. Despite the aforementioned consequences, low-income individuals have been compelled to seek out traditional medicines without taking into account how such medications may affect both their ability to reproduce and their developing fetus [44, 45].

1.2.Statement of the Problem

The WHO has made a significant involvement to the recognition and adoption of herbal treatment in African countries. In order to reduce the high death rate in the developing nations, between the year 202 to 2005, WHO recognized an agenda to support herbal therapies [22]. Despite the fact that many researches have shown the efficiency of African medicinal plants in treating a diversity of human ailments, there are still many unanswered questions, particularly with regard to their safety and quality control of these herbal medicines for the health of the general population [22, 46].

Studies showed that, substances derived from plant materials frequently have an impact on the survival and regular functioning of all animals, in one way or another. Such functional impacts might have either favourable otherwise unfavourable implications on the health status of the animals [27].

New medications are produced all around the world from medicinal plants [47-49]. In the developing nations, up to 80% of the population depend on herbal remedies for their main healthcare, and more than 25% of given medicines in industrialized nations derived from species of wild plants [47, 50]. Fast access to effective and safe medicines is of the utmost importance to diverse communities, patients, and consumers. One could assume that medicinal plants have little toxicity given the length of time that people have used them. Recent research, however, has shown that a number of traditionally used therapeutic herbs have negative effects [51, 52].

While literatures have recorded serious toxic effects from the use of herbs in many situations, their possible toxicity has not been acknowledged by the public or by professional organizations of traditional medicine. In addition, herbal species are typically regarded as harmless and shown to be beneficial against specific ailments [53].

In most studies, the toxicity of medicinal plants has been seen in blood's cellular and biochemical characteristics as well as in the histopathology of numerous internal organs. The most often identified organs to be impacted by the usage of medicinal herbs are the liver and kidney. There have also been harmful effects on the nervous system, cardiovascular system, blood and skin [54].

Almost all the Moringa plant's components have been used in the traditional remedy to cure a variety of diseases. This plant's fritted leaves are effective against fever and the common cold [55]. The anticancer properties of moringa leaves have been reported [56]. The seed extracts had a positive impact on mouse skin papilloma genesis, antioxidant parameters, and hepatic carcinogen metabolizing enzymes [57]. Niaziminin a thiocarbamate extracted from *M. oleifera* leaves (*M. oleifera*), has been revealed to suppress the activation of the Epstein-Barr virus caused by tumor promoters [58].

In general, conflicting information exists about the safety of various biological actions carried out on various Moringa plant components. In-vitro cytotoxic investigation showed that aqueous extract of *Moringa stenopetala* (*M. stenopetala*) seeds and leaves is harmless, however the ethanol extract includes harmful compounds that could be extracted using organic solvents or could be created through the extraction process [59]**.**

In addition, the teratogenic effects of the plant on the developing embryo and fetus have not been well investigated. Similarly, it is yet unclear how toxic the herb is to the reproduction. Therefore, the objective of the study was to examine the teratogenicity, reproductive toxicity, and developmental toxicity of *M. stenopetala* leaf in Wistar albino rats.

1.3. Significance of the Study

Studies shows that, kidneys, liver, nervous system, stomach and blood are just a few of the organs where herbal medication might have toxic effects [60]. As a result, the aforementioned organs are the subject of several toxicological studies. These studies demonstrate the tested plant's overall impact. However, amongst the utmost critical studies to be conducted on herbal remedies are their teratogenicity and reproductive toxicity profiles [27].

Researches on the reproductive effects of the Moringa species have shown contradictory findings. For instance, research on leaves from *M. oleifera*, which share ingredients with *M. stenopetala*, demonstrated an abortifacient effect and that its abortifacient potential occurs in the first trimester of pregnancy [61]. Furthermore, it was found that epididymis and seminiferous tubules of rats treated with *M. oleifera* showed tissue damage. However, according to other investigations, eating moringa leaves had no detrimental effects on male or female fertility indices and instead had favourable or boosting effects on the majority of reproductive parameters [62, 63].

Furthermore, the generation-based reproductive toxicity profile of *M. stenopetala* leaves has not yet been identified. In addition, the generations will suffer if a plant or one of its by-products has deleterious effects on parental reproduction. Consequently, the objective of the current investigation was to evaluate teratogenicity and reproductive toxicity of *M. stenopetala* leaf on Wistar albino rats. The results of this study may also be used to develop regulations governing the usage of this plant. Furthermore, the data generated can serve as a starting point for more in-depth toxicological studies in the future.

CHAPTER TWO

2. LITERATURE REVIEW

2.1.The "Miracle Tree," the Moringa plant

The plan Moringa makes up 10% of the estimated 250,000–500,000 plant species identified globally, and it has significant promise as a source of bioactive ingredients for the development of new drugs in the pharmaceutical sector [64]. The Moringa plant is indigenous to areas of Africa and Asia. It is the only genus in the family Moringaceae of flowering plants. Its name is derived from the word murungai [65].

There are several economic, nutritional, and therapeutic uses for the moringa plant in different parts of the world. All plant components belonging to this genus are employed in traditional human medical practices to cure a wide range of illnesses, and they are all rich in proteins, vitamins, minerals, and carotenoids. According to ethnobotanical studies, the leaves are used as purgatives to cure piles, headaches, ear infections, and eye infections, while the root is used to treat a variety of ailments like rheumatism and constipation [66].

There are currently 14 known species of the Moringa tree. *M. oleifera* is one of them that have been the subject of the greatest research into the discovery of chemicals with potential therapeutic use. Although this species is indigenous to India, it is also a common tree in Ethiopia [67].

This *M. oleifera* plant is nowadays known as a miracle or wonder tree in countries like Senegal [66]. Due to its numerous nutritional, medicinal, and industrial applications, it is employed to significantly enhance the socioeconomic and livelihood circumstances of the population [66, 68, 69]. The leaves of this plant are an excellent source of proteins, vitamins, beta-carotene, amino acids, and different phenolics. They also include a profile of significant trace elements. Various parts of this *M. oleifera,* including the root, bark, pods, and leaves, are used to cure human illnesses in the traditional medicine. The leaves are enhanced with vitamin A and C [70].

2.2.*Moringa stenopetala* (Baker f.) Cufod

Moringa stenopetala, which is a member of the Moringaceae family, is a fast-growing, domesticated Moringa species in East African. Because it grows in southern Ethiopia,

northern Kenya, and eastern Somalia, *M. stenopetala* is sometimes referred to as an African moringa tree [71, 72] (Figure 1). It is also present in Djibouti, Uganda, Sudan, South Africa, and Mozambique [72].

Figure 1: The map showing geographical distribution of *M. stenopetala* **[73].**

Moringa stenopetala is a native tree to Ethiopia, growing from 390 to about 2200 m above sea level in the Southern Rift Valley. It also thrives in semi-humid, semi-arid, and desert regions in the altitudinal range of 1,000 to 1,800 m above sea level [71]**.** It is a tiny tree that may grow up to 10 meters tall. It has a trunk up to 100 cm in diameter, which is bloated and bottle shaped. The bark is smooth and white, pale grey, silvery, or blackish. The crown is heavily branched. Young shoots are densely pubescent. The leaves alternate with 60 cm long panicles that have several flowers (Figure 2 and 3). It thrives in regions with temperatures ranging from 25 to 35 °C and yearly rainfall quantities of 250 to 1500 mm (millimetres). It can withstand temperatures as high as 48 °C in the shade. The major growing areas are the Arbaminch, Wolayta, Sodo, Konso, D'irashe, Gamogofa, Sidama, Bale, and Borana areas. *M. stenopetala* is known by several vernacular names. It is commonly referred to as Shiferaw in Amharic [74]**,** Halako in Gamo Gofa, Shelaqta in Konso, Haleko in Derashe, and cabbage tree in English. In southwest Ethiopia, the plant is grown as a traditional vegetable crop for human use. It is also used as a folk remedy for a variety of ailments, including malaria, hypertension, stomach pain, diabetes, cholesterol, spasms, and the expulsion of the retained placenta after childbirth [75-78].

Figure 2: *Moringa stenopetala* plant in the backyard of a farmer in southern Ethiopia **[78].**

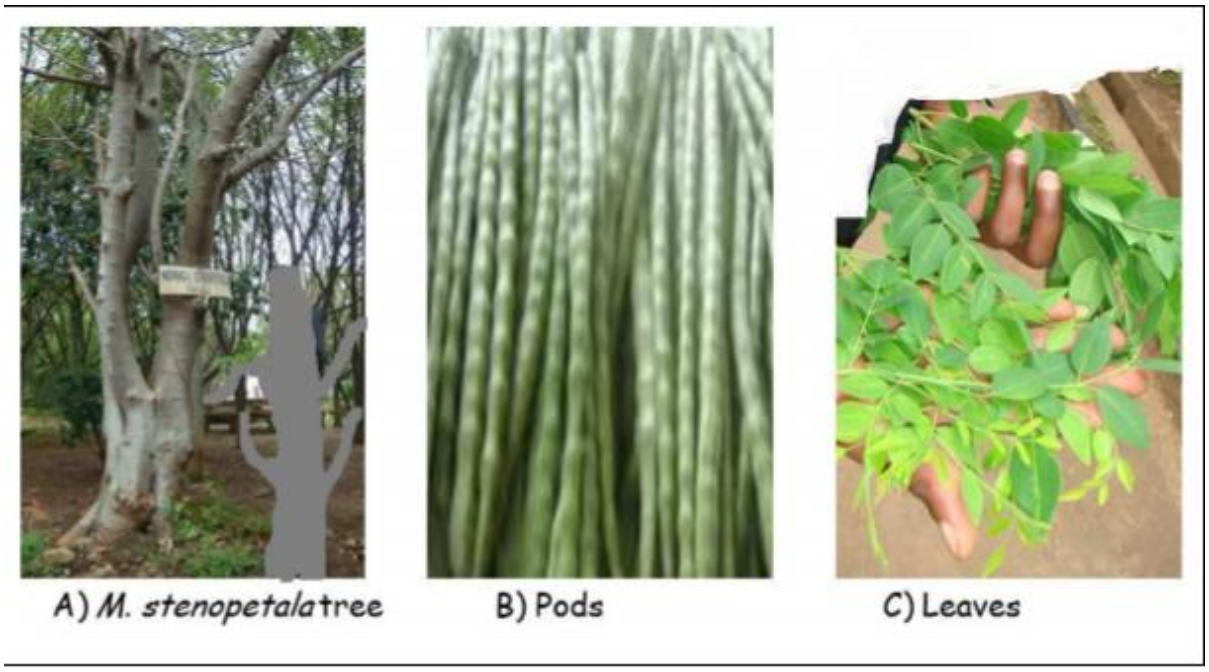


Figure 3: *Moringa stenopetala* tree, pods, and leaves **[79].**

People in southern Ethiopia consume the leaves as vegetables, especially during the dry season. Fruits are also consumed because they are nutritionally rich in proteins. Furthermore, the leaves and pods are utilized as animal feed. The seeds of *M. stenopetala* could flocculate, making them a promising cleansing agent in turbid water. Various plant

components have also been traditionally used to treat wounds, the common cold, and asthma [75-78, 80].

2.3. Traditional Applications of *M. stenopetala* Leaf

According to several scientific studies [81-83], the Moringa tree is believed to provide both nutritional and therapeutic benefits. *M. stenopetala* leaves, flowers, and green pods are used as a staple food and are a good source of protein, calcium, iron, and phosphorus.

Under smallholder farming methods in the tropics, *M. stenopetala* and several other native fodder plants and shrubs high in tannins are viewed as nutritionally prospective feed additions [84]. *M. stenopetala* is particularly vital as a food source for humans because of its nutritious leaves that emerge at the end of the dry season when few other sources of green vegetables are accessible [85]. The plant leaves might also be utilized as an animal and human food additive since they are loaded with nutrients [81]. In addition, Moringa is contains high quantity of antioxidants when compared to other fruits and vegetables. These comprise serving as protein, lipids, fiber, carbohydrate, and mineral resource feed compositions [86]. These revealed that the leaves of Moringa trees are an extremely beneficial source of nutrients for people of all age groups [87].

2.4.Phytochemical Property of *M. stenopetala* leaf

Many plants across the world, according to Griffiths *et al.* [88], contain secondary metabolites, some of which can operate as pollinator attractants, barriers against bacterial, viral, and fungal infections, and/or herbivore deterrents. Variety of secondary metabolites and other substances have been identified from several Moringa species [46]. The presence of pharmacologically useful chemical substances such alkaloids, cardiac glycosides, flavonoids, phenolics, phytosterols, saponins, tannins, coumarins, and terpenoids was discovered during phytochemical screening examination of *M. stenopetala* leaves. In addition, carbohydrates, proteins, lipids, and vitamins are among the functional molecules that have been found to have nutritional significance [89].

2.4.1. Alkaloids in *M. stenopetala* Leaf

The alkaloids are one of the biggest families of secondary metabolites found in plants, with at least 21,000 different structurally varied compounds found in 20% of all vascular plants

[90]. In accordance with the findings of Green *et al.* [91], alkaloids that can be generated from plants, plant products, or plant extracts might potentially result in developmental abnormalities in animals when they are exposed to developing embryos and fetuses.

2.4.2. Phenolics in *M. stenopetala* Leaf

The group of phytochemicals are known as phenolic compounds and these includes flavonoids, tannins, coumarins, quinones, and anthocyanins. For instance, flavonoids like rutin, which are found in some species of buckwheat (Fagopyrum esculentum), are known to decrease lipid oxidation by scavenging free radicals [90].

2.4.3. Terpenoids in *M. stenopetala* Leaf

Along with steroids, saponins, and cardiac glycosides, the terpenoids include monoterpenes, sesquiterpenes, sesterterpenes, and triterpenes. They are the phytochemicals with the broadest range of chemical compositions. The largest and most diversified family of natural products is the terpenoids, which range in size and structure from the five-carbon hemiterpenes to natural rubber, which contains hundreds of isoprene units [90].

2.4.4. Amino Acids in *M. stenopetala* Leaf

The leaf powders of *M. stenopetala* included a variety of amino acid compositions, including phenylalanine, valine, threonine, tryptophan, methionine, leucine, lysine, histidine, tyrosine, and isoleucine [89].

2.4.5. Vitamins in *M. stenopetala* Leaf

Six different kinds of vitamins, including thiamine (Vit B1), pyridoxine (Vit B6), niacin (Vit 3), folic acid (Vit B9), beta-carotene (Vit A), and ascorbic acid (Vit C), were found in *M. stenopetala* leaf powder. This evidence supports that the use of *M. stenopetala* leaves as a vegetable food and for nutritional purposes [89].

2.5.Safety Profile of *M. Stenopetala* Leaf

Access to quick, reliable, and effective therapies is of utmost importance to the diverse populations of patients and customers. Humans have used medicinal herbs for a long time, so one may assume they are harmless and non-toxic. Recent investigations, however, have

shown that many therapeutic herbs employed in conventional treatment have negative consequences [51, 52].

According to the knowledge gathered over eras, herbal plants are supposed to be harmless and their toxicological assessment has usually been ignored by many traditional healers [11]. Though, current scientific studies have revealed that many plants used as food or in traditional medicine are possibly toxic, mutagenic ,and carcinogenic [92, 93]. For instance, extensive usage of Scenecio, Crotalaria, and Cynoglossum in some regions of Ethiopia has been recognized as a likely factor in the development of chronic liver and kidney diseases [94]. However, it is a routine trend in Ethiopia for ordinary members of the local communities to use medicinal plants for their basic healthcare needs[95].

2.5.1. Toxicity of *M. stenopetala* Leaf

In a study conducted on the potential toxicity of the crude extracts and fractions of *M. stenopetala* leaves in rats, the highest dosages (1000 mg/kg/day) significantly reduced the rats' body weight gain compared to the normal control on day 15 of the experiment. Like this, prolonged administration of an ethanol extract from the leaves of *M. stenopetala* to diabetic control mice that had been exposed to alloxan resulted in an improvement in weight gain [60]. However, no acute toxicity of the test ingredient was seen in the experimental animals during a study to evaluate the effects of crude extracts and fractions of *M. stenopetala* leaves in normoglycemic and alloxan-induced diabetic mic [96].

2.5.2. Teratogenic Effects *M. stenopetala* Leaf

Developmental abnormalities can occur in both people and animals when pregnant women consume plants, plant products, or extracts that include the alkaloids piperidine, pyridine, or quinolizidine [91]. Teratogenic piperidine alkaloid-mediated desensitization of fetal muscle type is hypothesized to restrict fetal mobility in the developing fetus, leading to skeletal flexure abnormalities and cleft palate. The inhibition of fetal movement disrupts the normal developmental process to cause multiple congenital contracture-type (MCC-type) deformities like arthrogryposis, kyphosis, lordosis, scoliosis, and torticollis and cleft palate [91, 97, 98].

According to a study on the teratogenicity and embryotoxicity of *M. oleifera* extracts, that shares the same phytochemical composition as *M. stenopetala*, exhibited teratogenic

qualities in Zebrafish (Danio rerio). Low or absent heartbeat, development retardation, and morphological abnormalities of the embryos, such as yolk sac malformation and a shortened tail, were all indicators of these teratogenic characteristics [99].

Another teratogenicity and toxicity investigation using Wistar albino rat embryos and fetuses revealed that administering a crude extract of M stenopetala at a higher dose was unsafe for pregnant rats and caused a substantial delay in embryonic and fetal development. Additionally, it showed a decline in maternal weight gain throughout the course of the pregnancy as well as an uptick in fetal deaths and resorptions. A large dose of the plant extract also had negative effects on the placenta's histology, which showed intervillous thrombosis, decidual necrosis, and decidual hypoplasia [100]. However, most of the data necessary to evaluate developmental indices such as skeletal ossification centers and the morphology of internal organs are missing from this teratogenic investigation.

2.5.2.1.Placenta

The placenta is a feto-maternal organ that consists of two components. A maternal portion comes from the endometrium and a fetal portion develops from the chorionic sac. In terms of both anatomy and function, it is a complicated organ. According to the developmental stage and among species, the placenta's structure varies. Through its function as the lungs, intestines, kidneys, hematopoietic gland, and endocrine/exocrine gland, it significantly contributes to the maintenance of pregnancy and the growth of the fetus. It serves as the main location for the mother and fetus to exchange nutrients and gases. Through the placenta, nutrients and oxygen are transferred from the maternal blood to the fetal blood, while waste products and carbon dioxide are transferred from the fetal blood to the maternal blood. In general, the placenta and the fetal membranes carry out functions related to defence, feeding, respiration, excretion, and hormone synthesis (Figure 4). The placenta and fetal membranes are evacuated from the uterus shortly after delivery, as after birth [101].

Additionally, it allows chemical information to pass between the mother and fetus [101]. The placenta is extremely vulnerable to the effects of toxins because of how it works [125]. In addition to maternal exposure to chemicals, several of these substances can cross the placental barrier and reach the embryo or fetus. As a result, the chemicals may have an impact on the mother herself, the placenta, and even the fetus [126]. Exposure of pregnant

animals to chemical substances during the early embryonic phase may cause problems with the differentiation of trophoblast cells into the placental membrane [127], as well as with the development of embryonic tissue [128].

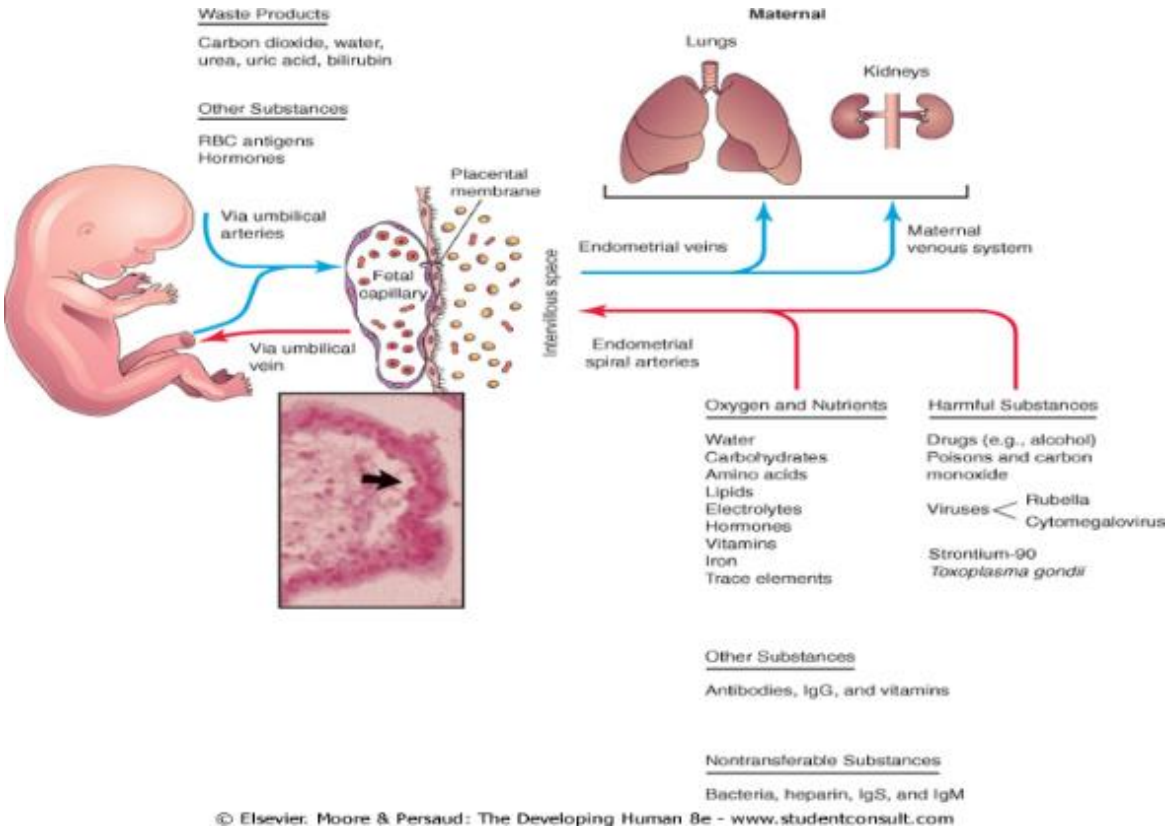


Figure 4: Diagrammatic illustration of transfer across the placental membrane [101]

2.5.3. Reproductive Toxicity of Moringa

Reproduction is an indisputable requirement for life and is crucial to human survival. Different kinds of food or nutrition can be used by people to control their reproductive function, which is a crucial aspect in controlling the reproductive function of other creatures [102]. Consequently, several therapeutic herbs have active ingredients that could encourage animal reproductive [103-105].

Changes in sperm motility or morphology as well as a drop-in sperm count are considered when discussing male reproductive toxicity. The primary male sex organ is the testis. Histologically, it is made up of tightly packed convoluted loops of seminiferous tubules that are separated by an interstitium that has Leydig (interstitial) cells, vasculature, macrophages, a protein-and testosterone-rich ultrafiltrate, and supportive stroma. The basally positioned Sertoli cells that make up the seminiferous epithelium sustain

successively synchronized populations of growing germ cells, such as spermatogonia, spermatocytes, spherical spermatids, and elongating spermatids [106, 107]. One of the most sensitive markers of injury is the histopathology of the testicles, which may or may not be accompanied by a change in the functional outcome of mating [108]. The testis should be examined histopathologically to check for retained spermatids, missing germ cell layers or kinds, multinucleated giant cells, and spermatogenic cells that have spilled into the lumen [106].

Seminiferous tube fluid, which contains the sperm, is transported from the testis through the rete testis, efferent ducts, and the first segment of the epididymis. In the rat, the epididymis is a convoluted tube that is about 180 cm long. Depending on where it is located, the epididymis's cellular composition, epithelial height, ductal diameter, and sperm density can all differ. The epididymis is where sperm are modified, mature, and stored in addition to where the seminiferous tubular fluid is reabsorbed [109]. When looking at the intact epididymis, it should include the caput, body, and cauda. In addition, histopathologically, it should be evaluated for leukocyte infiltration, sperm granulomas, change in prevalence of cell types, change in constitutive cells like clear cells in the cauda epithelium, aberrant cell types in the lumen and phagocytosis of sperm cells [106].

In the study conducted to investigate the beneficial effects of *M. oleifera* leaf on the reproductive performance of mice, the plant extract showed a significant increase in the weight of the testis, seminal vesicle, and epididymis. Furthermore, the plant extract exhibited an increase in the diameter of the seminiferous tubule and a higher score of epididymal maturity and light formation [110]. In addition, the *M. oleifera* leaf also exhibited protective activities in spermatogonia cells and reduced cell damage in mice injected with cyclophosphamide [110, 111]. Furthermore, the hexane extract from *M. oleifera* improved the functions of the seminiferous tubule, epididymis, testes, and seminal vesicles in male mice [112].

According to the report of Ogunsola OA, *et al*., administration of Moringa leaves at 200mg and 500mg per kg body weight for 30 days significantly increased the number of implantation sites on day 8 of pregnancy in rats. On the other hand, the site of implantation was not observed in the high and low-dose group of rats administered with other Moringa plant parts namely the seed, flower, root, and stem. They suggested that consuming Moringa leaves increases reproductive function and fertility in rats because the number of

embryos was increased and successfully implanted into the uterus of rats. They also reported that consuming Moringa leaves can improve reproductive function in females by increasing the secretion or availability of ovary hormones, including progesterone and estrogen. Progesterone is traditionally known to be essential for pregnancy maintenance as it keeps the uterus quiescent preventing the premature onset of labor, hence its name "pro-gestation", while estrogens are essential for ovulation, implantation, pregnancy maintenance, and childbirth. This result also means that the consumption of Moringa leaves is harmless and does not cause abortion during pregnancy. However, the consumption of the other parts of the Moringa plant, namely the seed, the flower, the root, and the stem, is not recommended, as they may be anti-fertility or abortive [62].

Matching with the above reports, lowered rate of abnormal sperm cells in *M. oleifera* leaf-treated groups than in the control group was reported by Zeng, B., *et al.* [110], and the finding demonstrated that the plant could improve semen quality in mice. As well, the plant had no significant effect on serum testosterone levels in male mice and serum estradiol levels in female mice. Similarly, the plant extract does not affect serum concentrations of FSH and LH [112].

On the other hand, reports of other studies revealed that alkaloid, which is one of the biochemical components of Moringa, is known to have an adverse effect on pregnancy and is being used by physicians either alone or in combination with oxytocin to induce abortion [113]. Furthermore, the anti-fertility and abortive activities of phenolics, phytosterols and saponins that are constituents of *M. stenopetala* have been reported in animal models [114].

However, there is limited information on whether *M. stenopetala* enhances reproductive performance in animals or is toxic to reproductive organs. Thus, this study aimed to investigate the teratogenic effects of ethanolic extracts of *M. stenopetala* leaf on the developmental status of embryos and fetuses of Wistar albino rat. In addition, the study was designed to assess the effects of the plant on developmental landmarks, reproductive parameters, serum parameters, and the histopathology of parent (F0) and first-generation (F1) male and female reproductive organs.

CHAPTER THREE

3. OBJECTIVES of the STUDY

3.1.General Objective

- The general objective of the current study was to evaluate two-generation reproductive toxicity and potential teratogenicity of 70% ethanol extract of *M. stenopetala* leaf in Wistar Albino rats

3.2.Specific Objectives

The specific objectives in this study were:

1. To evaluate the possible teratogenic potential of *M. stenopetala* leaf on the developing rat embryos at the age of 12 days
2. To investigate the possible teratogenicity of *M. stenopetala* leaf on the developing rat fetuses at late pregnancy on gestation day twenty
3. To examine the effects of *M. stenopetala* leaf extracts on the histopathology of the placenta of rat fetuses at gestation day twenty
4. To investigate the effects of *M. stenopetala* leaf on reproductive indices of F0 (Parents) and F1 (first generation) parental rats
5. To evaluate the effects of *M. stenopetala* leaf on developmental parameters of F1 and F2 (second generation) pups
6. To investigate the effects of *M. stenopetala* leaf on the histopathology of reproductive organs of F0 and F1 parental animals
7. To evaluate the effects of *M. stenopetala* leaf on the reproductive hormones of F0 and F1 parental rats
8. To assess the effects of *M. stenopetala* leaf on the serum profiles of F0 and F1 parental rats

CHAPTER FOUR

4. MATERIALS and METHODS

4.1. Study settings and Experiments

The current study was carried out in the laboratories of Ethiopian Public Health Institute (EPHI), Traditional and Modern Drug Research Directorate (TMDRD) and departments of Anatomy and Pathology of AAU. This study included two major experiments. The first study looked at the potential toxicity and teratogenicity of ethanol extracts of *M. stenopetala* leaf on the developing rat embryos and fetuses following exposure of the pregnant rats during critical period of organogenesis and embryogenesis. The second experiment was evaluated reproductive toxicity of the plant on the two-generation of male and female rats.

4.2. Study Period

The reproductive and developmental toxicity study was conducted from December 2018 in G.C. to September 2021.

4.3.Validity and Reliability of the measurements

All of the study's machinery, supplies, and reagents were thoroughly examined to ascertain their validity and reliability before the actual commencement of the experimental procedures and data collection processes.

4.4.Plant Material Collection and Preparation of the Extracts

As shown in figure 5, fresh leaves of *M. stenopetala* were collected in the southwestern region of Ethiopia., around Arbaminch city, which is 500 kilometres farm Addis Ababa, the capital city of Ethiopia. The plant was recognized and identified in the EPHI by a senior researcher, where a voucher number AL-001 was given and deposited in the herbarium for the use and future reference [115]. In the laboratory the leaves were cleaned from dust particles, washed using tap water, mangled and sliced into small pieces and then dried under shade, finally grounded to powder using an electric mill and deposited at room temperature. To get the crude extracts, the powder was mixed with 70% ethanol in the ratio of 1:10 powder to solvent in an Erlenmeyer flask and wrapped using aluminium foil and

then placed in an orbital shaker and rotated for 24 h at 100 revolutions per minute (RPM). Then filtrate was obtained by filtering the solvent using Whatman No, 1 filter paper (18 cm in diameter). Using a rotary evaporator, (R-205, Büchi Rota Vapor, product of Switzerland) at 175 millibar pressure and temperature of 40°C. The crude extract was the obtained by drying the filtrate in a hot water bath at a temperature of 45°C. Finally, the filtrate was placed in a wrapped glass container and stored at -20°C in a refrigerator until utilized during the experimental procedures [116].

Figure 5: Plant material preparation and extraction using 70% ethanol

4.5.Preparation for the Teratogenicity Test

For teratogenicity investigation in the current study, two experimental designs were employed. The first experiment is called day 12 experiment that was designed to examine the possible teratogenic effects of 70% ethanol extracts of *M. stenopetala* leaf on 12 days old rat embryos. The second experiment is called day 20 experiment that was designed to investigate the teratogenic potential of 70% ethanol extracts of *M. stenopetala* leaf on near term rat fetuses at day 20 of the gestation period.

4.5.1. Experimental Animals

In this investigation, healthy, virgin Wistar albino rats, that were 10 to 12 weeks old, weighing 225 to 40 g were used as experimental and control groups. They were obtained from animal breeding unit of EPHI and placed in the animal house of TMDRD. Before the commencement of the actual experiment, the animals were inspected for the presence of any health problems and acclimatized to the environment for one week.

The animals were placed in an environmentally controlled room with temperature of 23±3°C, relative humidity of 50±10%, and an alternating 12 h dark and light cycles. During the adaptation period, the animals were fed a standard pellet with composition of 16% protein, 75% carbohydrate, 55% fat, 3.6% calcium, and 0.4% phosphate with free access to tap water *ad libitum* (Figure 6).

Figure 6: Pregnant rat placed in stainless steel cage and feed a standard pellet

In this study, following one-week adaptation, one randomly selected male rat with confirmed fertility was placed in a stainless-steel cage containing one virgin female rat. After an overnight mating, the female rats were inspected to determine whether a copulatory plug had formed, and a vaginal smear was done and observed under microscope for the presence of sperm cells (Figure 7). The day when the sperm cells detected in the vaginal smear was considered gestation day one (GD 1) [117].

Figure 7: Inspection of female rats for the presence of copulatory plug following an overnight mating with male rat.

4.5.2. Experimental Design

Fifty pregnant rats were used for each experiment; their identifying numbers were permanently inked on their tails, and they were split into five groups of ten animals each (Tables 1 and 2). *M. stenopetala* leaf extract was administered orally to the first three experimental groups (groups I to III). They were given 250 mg/kg, 500 mg/kg, and 1000 mg/kg of body weight of the plant extract, respectively. According to the OECD recommendations 416 and 421, at least three dose levels should be used, which is why the concentration doses listed above were selected [118-120]. Dosage levels should also be selected with consideration for any available toxicological data, especially results from repeated dose studies. It's important to consider the test compound's metabolism and kinetics as well as data on related substances. Consequently, the doses chosen in this study considered 10% of LD50 of *M. stenopetala* leaf extract, which is more than 5000 mg/kg as reported from previous efficacy study [60]. The other two doses such as 250 mg/kg and 1000 mg/kg were half above and below the 10% of the LD50 of the plant extract. therefore, 500 mg/kg is 10% of the 5000 mg/kg. This led to the selection of 250 mg/kg was half below the 10% of the LD50 and the highest dose which is 1000 mg/kg was double of the 10% of the LD50 of the plant. The other two groups pair-fed and ad libitum control groups. The fourth group was administered distilled water at a volume of 2ml/100 g body weight of the animals and fed with an amount that was matched to the volume consumed by the experimental groups. The fifth group was an ad libitum control that was remained untouched throughout the experiment and was fed freely. The extract was administered in

both experiments from gestation days 6 through 12, when organogenesis and embryogenesis were actively taking place. Every morning, the amount of food that each animal consumed was recorded. Animals were weighed on gestation days of 1, 6, and 12 for the first teratogenicity experiment that is day-12 experiment and on gestation days of 1, 6, 12, and 20 for the second teratogenicity study designed to investigate teratogenicity potential on near term fetus at gestation day 20. It was determined how much weight gained during each interval. The rats were observed daily to ensure that the treatment was not having any negative effects. Throughout the experiment, the experimenter took unclear notes without knowing which rats were the control and treated group [121, 122].

4.5.3. Day-12 Experiment

The aim this experiment was to examine embryotoxicity of 70% ethanol extract of M. stenopetala leaf on 12 days old rats. Due to the potential compensatory growth and development, this study aimed to investigate any retarded growth and developmental anomalies that could not been observed in the late pregnancy, and near-term rat fetuses that would have been born shortly.

Table 1: Treatment schedule for day 12 experiment

Animal grouping	No of rats	Descriptions of the grouping
Group I	10	250 mg/kg b.w EtOH extract of M. stenopetala leaf plus food and water
Group II	10	500 mg/kg b.w EtOH extract of M. stenopetala leaf plus food and water
Group III	10	1000 mg/kg b.w EtOH extract of M. stenopetala leaf plus food and water
Group IV	10	Distilled water (2ml/100 g body weight) plus matched food and water
Group V	10	Unrestricted food and water

- b.w: body weight; EtOH: ethanol

After administration of the extract for 7 days, from gestation day 6 to gestation day 12, (day 12 experiment), at 12:00 o'clock, the pregnant animals were sedated by injection of pentobarbital sodium intraperitoneally at a dose of 150 mg/kg of the body weight of the animals [123]. Using an incision, the abdomen was opened, the horns of the uterus were

removed and put in a glass container filled with normal saline, and incised on the antimesometrial border to reveal the embryos. The yolk diameter was measured and the membranes enclosing the embryo were cut away and the visceral yolk sac was exposed using a fine extractor and a dissecting microscope (Figure 8).

The developmental status of the embryos in the current study was evaluated with help of a dissecting microscope. Development of the allantois and yolk sac circulation were assessed. Correspondingly, the developmental status of the organ systems was evaluated following the morphological rating system of Brown and Fabro [124] that was adopted for the in vivo toxicological study by Seyoum and Persaud [125]. Subsequently, the embryos' craniofacial development, skeletal system, nervous system, and special sense organs (olfactory, auditory, and visual) were assessed for development. Furthermore, the CRL of the embryo was measured and the somite number was counted.

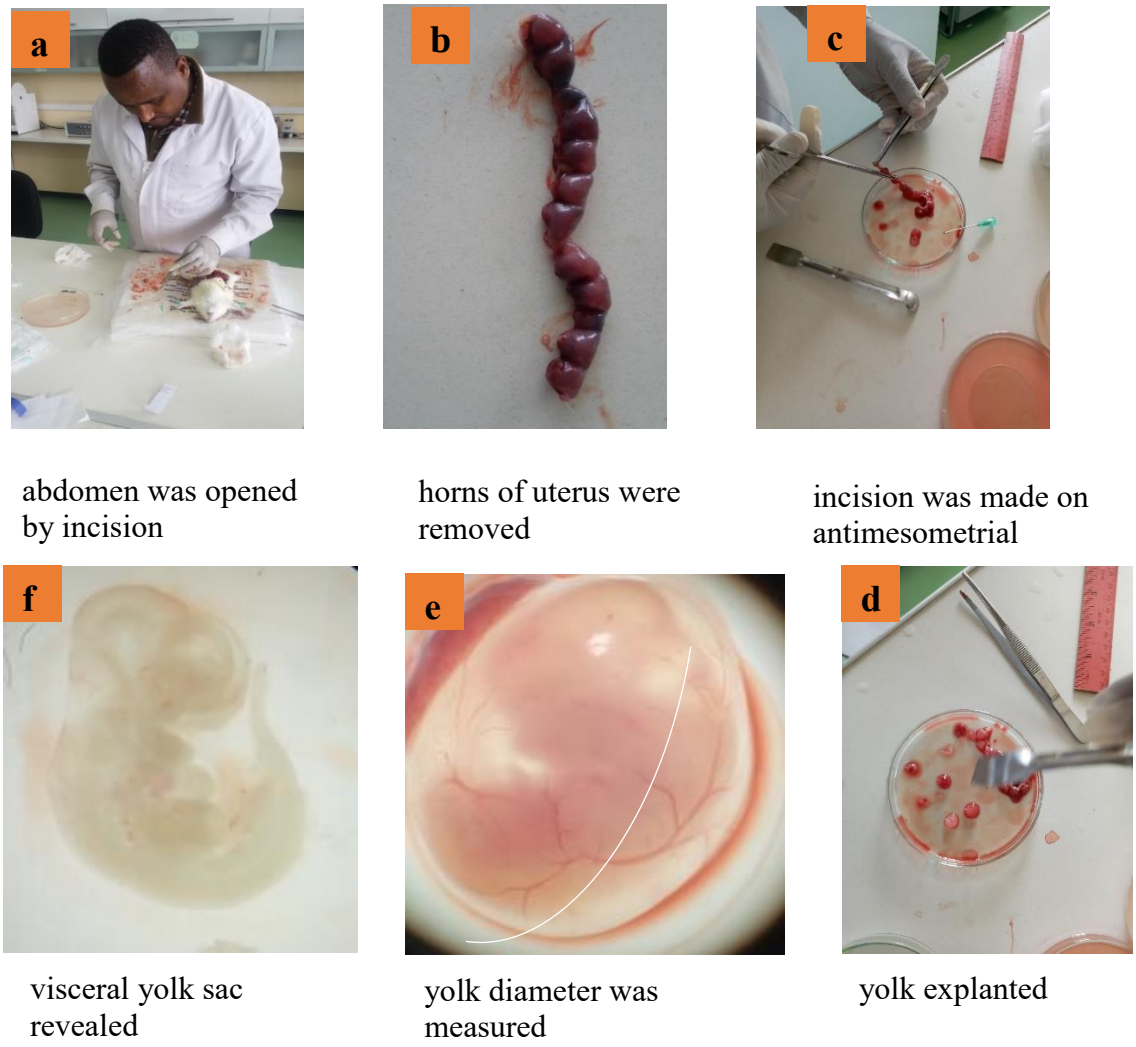


Figure 8: Figure shows experimental procedure in day 12 experiment

4.5.4. Day-20 Experiment

This day 20 experiment was designed to assess the *in-vivo* toxic effect of 70% ethanol extract of M. *stenopetala* leaf on 20 days old fetuses and placentas of pregnant Wistar rats. It investigated the developmental toxicity of 70% ethanol extract of *M. Stenopetala* leaf in near-term fetuses and histopathological changes of the placenta.

Table 2: Treatment schedule for day 20 experiment

Groups	**No. of animals**	**Descriptions**
Group I	10	250 mg/kg b.w EtOH extract of *M. Stenopetala* leaf plus food and water
Group II	10	500 mg/kg b.w EtOH extract of *M. stenopetala* leaf plus food and water
Group III	10	1000 mg/kg b.w EtOH extract of *M. stenopetala* leaf plus food and water
Group IV (Pair-fed control)	10	Distilled water (2 ml/100 g body) plus matched food and water
Group V (*Ad libitum* control)	10	Unrestricted food and water

- b.w: body weight; EtOH: ethanol

The animals were humanely killed on gestation day 20 after being sedated with pentobarbital sodium. The following features were assessed: the success of the pregnancy, the morphological, skeletal, and visceral evaluations, and the placental histopathological studies.

4.5.4.1.Pregnancy Outcomes Evaluations

The number of implantation sites, resorption sites, live fetuses, dead fetuses, and sex of the fetuses were counted in order to assess the pregnancy outcomes. The pregnant rats' anterior abdomens were opened, exposing, removing, and examining their uterine horns in order to assess these parameters. A yellow-colored nodule, which is the metrial glands found at the border of the mesometrial of the horns of the uterus, was counted in order to determine the number of implantation sites. The metrial nodules that were free of live or recently

deceased fetuses were indicated early resorptions of the fetuses. By applying light pressure, the number of live or dead fetuses was ascertained. After being extracted, the uterine horns were cut along their anti-mesometrial borders and placed in a sterile glass container. Each fetus was immediately exposed, separated from the placenta, and the weight of each fetal membrane was recorded. The weight of the placenta was noted. Every fetus's weight and CRL were measured, and their sex was determined.

In this study, dead or live fetuses were identified by applying a mild pressure on them. The horns of the uterus were removed and placed in a clean glass container and then incised along the anti-mesometrial border of each horn. Instantly, each fetus was revealed and detached from the placenta and all fetal membranes were weighed. The placental weight was recorded. The sex of the fetuses was identified, and the weight and CRL of each fetus were measured (Figure 9). The procedures followed the methods described by Seyoum and Persaud [125] that were adopted for the *in vivo* toxicological study from Brown and Fabro [124].

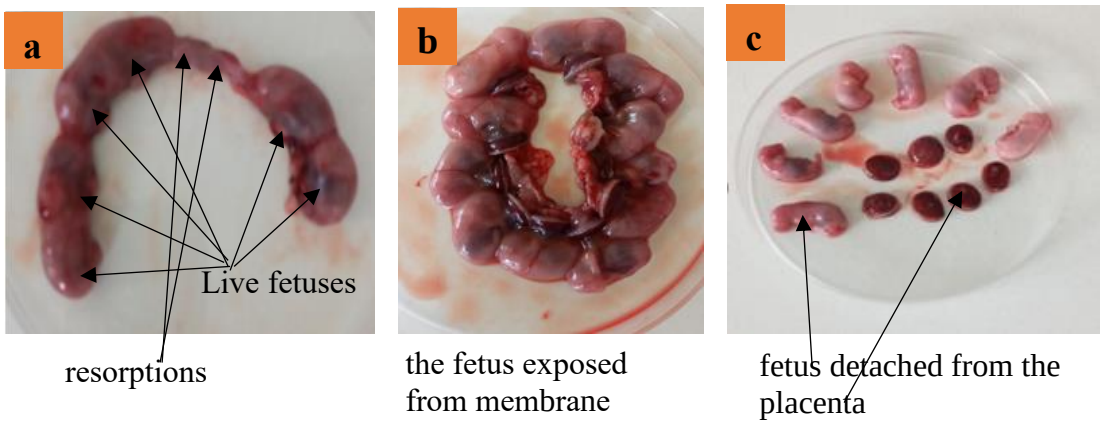


Figure 9:Figure shows fetuses in the uterus (a), exposed from membrane (b) and detached from placenta (c) in day 20 experiment

4.5.4.2.Morphological Evaluation on Day-20 Rat Fetuses

Once each fetus was revealed and separated from the corresponding placenta, all fetuses were examined from head to tail for the presence of any gross developmental malformations. The assessment includes craniofacial anomalies (exencephaly, anencephaly, microphthalmia and anophthalmia); limb abnormalities (polydactyly, syndactyly, adactyly); anomalies of vertebral column (kyphosis, neural tube defect, scoliosis), a disorder of development of tail like missing of tail; and malformation of

external genitalia. For examination of skeletal developmental delays, 2 to 3 fetuses from each litter were randomly chosen. The rest of the fetuses in the study were placed and fixed in Bouin's solution for the period of 2 weeks (25% formalin, 5% glacial acetic acid and 75% picric acid) for visceral examination [124].

4.5.4.3.Visceral Examination on Day-20 Rat Fetuses

Following gross external examination of the fetuses, visceral organs were examined by serial sectioning that was made on the body of fetuses fixed in the solution of Bouin's for 2 weeks. Following a Modified Wilson technique, the fetuses were sectioned using a surgical lade [126, 127].

The sections were done craniocaudal at intervals of 1–2 mm with the help of dissecting microscope (XTL3101, 6 × magnifications). Beginning at the jaw, the sectioning was carried out dorsally superior to the ear. After the tongue was extracted, the palate was checked for any clefts. Furthermore, a transverse section of the neck and parts below, as well as a coronal section of the head, were completed in order. The brain (hydrocephalus, dilation of ventricles, microphthalmia/anophthalmia), the craniofacial region (cleft palate, nasal septum defect), the thoracic region (lungs, heart, retro-oesophageal aortic arch, septal defect), the abdominal region (liver, stomach, and gut anomalies), and the pelvic region (kidneys, ectopic kidney, and hydronephrosis, gonads: testes and ovarian anomalies) were examined for any visible abnormalities [128].

4.5.4.4. Staining of Skeleton and their Evaluation

Skeletal staining was done by employing the method of Dawson [129-131]. Depending on the litter size, 2–3 fetuses per litter were randomly selected and killed by an overdose of pentobarbital. These fetuses were eviscerated, and all internal organs were taken out through incision made on the median plane of the anterior part of abdominal wall. Then, they remained and kept in a small plastic flask that filled with 95% of ethanol and the fluid was dehydrated from the fetuses for the period of one week.

After dehydration, the delicate tissues of the specimens were cleared for 2–3 days in 1% KHO solution until bones of the specimen were clearly observed. At that moment, the specimens were relocated to a new solution of 1% KOH and were stained with a few drops that was a 0.4 ml of alizarine red. The specimens were stained overnight, and the

overstaining of the specimen was adjusted by placing the specimens in Mall's solution a mixture of 20% glycerine, 79% of distilled water, and 1% of KOH. Then, the specimens passed through rising concentrations of glycerine (20%, 40% 60%, and 80%) for the period of one week in each of the concentration. Lastly, specimens were placed in 100% glycerine for evaluation. In addition, a trivial thymol crystal was dropped in to the specimen to prevent growth of fungus prevent the specimen.

Finally, each specimen was seen under a dissecting microscope with transparent background and brightfield optics. The degree of ossification of the primary indicators for skeletal maturity in rodents, such as the metatarsal, metacarpal, sternebrae, and sacrococcygeal bones, has been evaluated [130]. The extent and quantity of ossifications centers in each bone were studied with help dissecting microscope. All bones of the limbs, the skull, ribs, sternebrae, hyoid bone, and vertebrae were carefully assessed. The assessment was conducted using a skeletal scoring chart designed by Nash and Persaud [132]. After investigation, sample photomicrographs were captured using digital dissecting microscopic camera (XTL3101, England) underneath 4× magnifications.

4.5.4.5.Placental Examination of Day-20 Rat Fetus

From all groups, each placenta was examined for any gross morphological abnormalities. Furthermore, two to three Placentae/dam/groups were randomly selected for histopathologic examination. From each placenta, a sample in the size of 3 to 4 mm was taken, and it was fixed by dipping it in 10% formalin. The tissues were dehydrated in an increasing concentration of alcohol (40%, 50%, 70%, 80%, 90%, and 100%) after fixation of the them for a full night. The tissues were then cleared using three steps of xylene (xylene I, xylene II, and xylene III). After clearing, the placental tissues were infused with melted paraffin wax (I and II). Finally, each sampled tissue was placed in an embedding cassette and filled with melted wax. A 5 µm section was made for every block and the ribbon was placed on the frosted slide and then kept for 20 to 30 minutes in a hot oven with temperature ranging 40 to 45 °C [133].

The following steps were applied to stain the placental tissues: the paraffin wax was removed from the slides using three steps of xylene for five minutes in each of the three xylenes, then rehydrated in a decreasing concentration of alcohol from absolute alcohol I and II, to 90% alcohol, 80% alcohol, and finally to 70% of alcohol for two minutes in each of the concentration. Then the slides were washed using running of a tap water for

two minutes. After that the slides were stained using haematoxylin for 5 to 10 minutes, then the slides were cleaned with a running tap water for 10 minutes, then deep in acid alcohol for 2 to 3 seconds, and counterstained using eosin for 1 to 2 minutes. Furthermore, the stained slides were dehydrated in an increasing concentration of alcohol starting from 80%, 95%, and to absolute alcohol I and II for two minutes in each of the concentrations. Then the slides were cleared in xylene I, II, and III, two minutes in each of the xylene steps. Lastly, after clearing, the slides were mounted using DPX and shielded using a coverslip [71]. In the stained slides, a senior pathologist investigated the structural integrity of the placenta using a binocular light microscope. The decidual zone, the labyrinthine zone, giant cells, and trophoblasts of the placenta were investigated. Sample photograph of the placental tissues were captured using an automated digital microscopic camera (Leica EC4, product of Germany) underneath 10× and 40× objective lens magnifications.

4.6.Evaluation of Two-generation Reproductive Toxicity

4.6.1. General Description

The goal of this two-generation reproductive toxicity evaluation was to identify the effects of *M. stenopetala* leaf extract on the reproductive parameters and biochemical profiles in the serum of male and female parental (F0) and first generation (F1 generation) rats. In addition, this research intended to investigate the effects of *M. stenopetala* leaf extract on the developmental and growth parameters of F1 generation and second generation (F2 generation) offspring. In the study, assessments of weight, sperm parameters, estrus cycle parameters, and offspring parameters were included. It also included assessments of histopathology, gross morphological structures, and daily clinical observations in parental rats of both male and female F0 and F1 rats. Furthermore, the study evaluated the effects of *M. stenopetala* leaf extract on the reproductive function, parturition of animals, lactation, and postnatal development of the pups, as well as growth status and sexual maturation pups of both F1 and F2 generations.

4.6.2. Experimental Animals

In this reproductive toxicity study of two-generation, nulliparous female and young male Wistar albino rats, weighing 210–230 g and ages 5–9 weeks, were gained from the Ethiopian Public Health Institute animal breeding department and utilized as F0 parent

animals. The animals were then kept for the experiment in TMMRD's animal room. In the study, animals in a good health conditions were selected for use [118-120].

4.6.3. Grouping and Dosing of Animals

In this reproductive toxicity study of the two-generation, the dosage selections and animal grouping were similar to those used in the teratogenicity study, with the exception that in the reproductive toxicity study 20 rats were used in each group of the F0 and F1 parental animals. The animals were also adapted to the test centre for one week.

After one-week acclimatization, two hundred rats (100 male and 100 female rats) were randomly grouped into three treatment and two control groups. In each group 20 animals were assigned. The first three groups (G I to III) were given *M. stenopetala* leaf extracts orally at doses of 250 mg/kg, 500 mg/kg, and 1000 mg/kg body weight. The fourth group (IV) was the pair-fed control group that was gavaged 2 ml/100 g body weight of distilled water and was fed with a volume that was adjusted to the amount consumed by the experimental groups. The fifth (V) group was an *ad libitum* control that was fed freely and remained untouched throughout the experiment (Table 3). An intragastric tube was used to administer the extracts. The same procedure was used to treat all of the rats.

Table 3: Treatment schedule for reproductive toxicity study for F0 parental animals

Groups	**No. of animals**	**Descriptions**
Group I	40 (20 M & 20 F)	250 mg/kg b.w EtOH extract of *M. Stenopetala* leaf plus food and water
Group II	40 (20 M & 20 F)	500 mg/kg b.w EtOH extract of *M. Stenopetala* leaf plus food and water
Group III	40 (20 M & 20 F)	1000 mg/kg b.w EtOH extract of *M. stenopetala* leaf plus food and water
Group IV (Pair-fed control)	40 (20 M & 20 F)	Distilled water (2 ml/100 g body) plus matched food and water
Group V (*Ad libitum* control)	40 (20 M & 20 F)	Unrestricted food and water

- M: male; F: female; b.w: body weight; EtOH: ethanol

4.6.4. Experimental Design and Mating Procedures

The daily treatment of the F0 parental animals commenced at the age between 5 to 9 weeks, whereas the F1 parental rats' daily dosing began at weaning. Both sexes received treatment for 10 weeks of premating period. In addition, the dosage was continued for the additional two-weeks of mating period, when female rats were housed in cages with male rats until pregnancy was confirmed. Later, male rats were humanely killed to assess male reproductive function. It is intended to evaluate the effects of the plant on the spermatogenesis process by administering the extract to the animals for 10 weeks throughout the premating stage that included full period of spermatogenesis also further during the 2 weeks of mating period. The female animals were dosed for the 3 weeks of pregnancy and the 3 weeks of additional lactation time (Figure 10). Except during mating, male and female rats were placed apart. The pregnant rats were each placed in a separate maternity cage. After weaning, those F1 pups that were not selected for mating and F2 offspring that were not selected for the study of sexual maturation had humane autopsies [118-120].

4.6.4.1.Parental (F0) Mating

The F0 rats were mated over night by placing one male rat in to a stainless-steel cage that contain one nulliparous female rat at a 1:1 ratio. Following an overnight placement of male and female rats, the next morning, female rats were assessed for the existence of a vaginal plug and a vaginal smear was taken to evaluate the presence of spermatozoa using microscope. The pregnancy was confirmed after checking spermatozoa inside the vaginal smear. The first day of pregnancy (GD 1) was considered following detection of spermatozoa in the smear from vaginal plug [117]. The mated F0 female rats were kept individually during the 3 weeks of pregnancy whereas with their F1 pups in the same cage for an extra three weeks of lactation period [118-120]. The delivery date of the dam was used as PND 0. On PND of 4, more than eight pups were corrected by randomly removing additional pups, resulting in each litter having four males and females each. The F1 puppies were chosen at random on PND 21 to produce the next generation. They were then kept in four rats per sex per group and given the same dosages as their parents from the time of weaning until sacrifice.

4.6.4.2.F1 Mating

To produce F2 generation, the F1 offspring—one male and one female rat—were selected at weaning from each litter for mating with other pups of the same dose level from different litters. Mating of Siblings was avoided. The F1 offspring were mated at the end of the premating period of 12 weeks when they attained full sexual maturity. The dosing of the F1 parental rats was like that of the F0 parental study (Table 4 and Figure 10).

Table 4: Treatment schedule for reproductive toxicity study of F1 parental animals

Groups	**No. of animals**	**Descriptions**
Group I	40 (20 M & 20 F)	250 mg/kg b.w EtOH extract of *M. Stenopetala* leaf plus food and water
Group II	40 (20 M & 20 F)	500 mg/kg b.w EtOH extract of *M. Stenopetala* leaf plus food and water
Group III	40 (20 M & 20 F)	1000 mg/kg b.w EtOH extract of *M. Stenopetala* leaf plus food and water
Group IV (Pair-fed control)	40 (20 M & 20 F)	Distilled water (2 ml/100 g body) plus matched food and water
Group V (*Ad libitum* control)	40 (20 M & 20 F)	Unrestricted Food and water

4.6.5. Clinical Observations

During the entire experimental period, all animals were inspected daily for the presence of clinical signs and abnormal behavior. The body weight of male and female animals was recorded on a weekly basis for 10 weeks of premating and 2 weeks of mating periods. Furthermore, the body weight of mated female rats was measured on gestation days 0, 7, 14, and 21 and during lactation on PND 1, 7, 10, 14 and 21. The animals were weighed on the date of necropsy and relative organ weight of testis, epididymis, prostate gland, and seminal glands in males, as well as uterus and ovary in females, was calculated. During the premating period, food consumption was assessed every week. Food intake was monitored for mated females from gestational days 0-7, 7-14, and 14-21 and from postpartum days 1-7, 7-14, and 14-21 for females with live pups [118-120, 134].

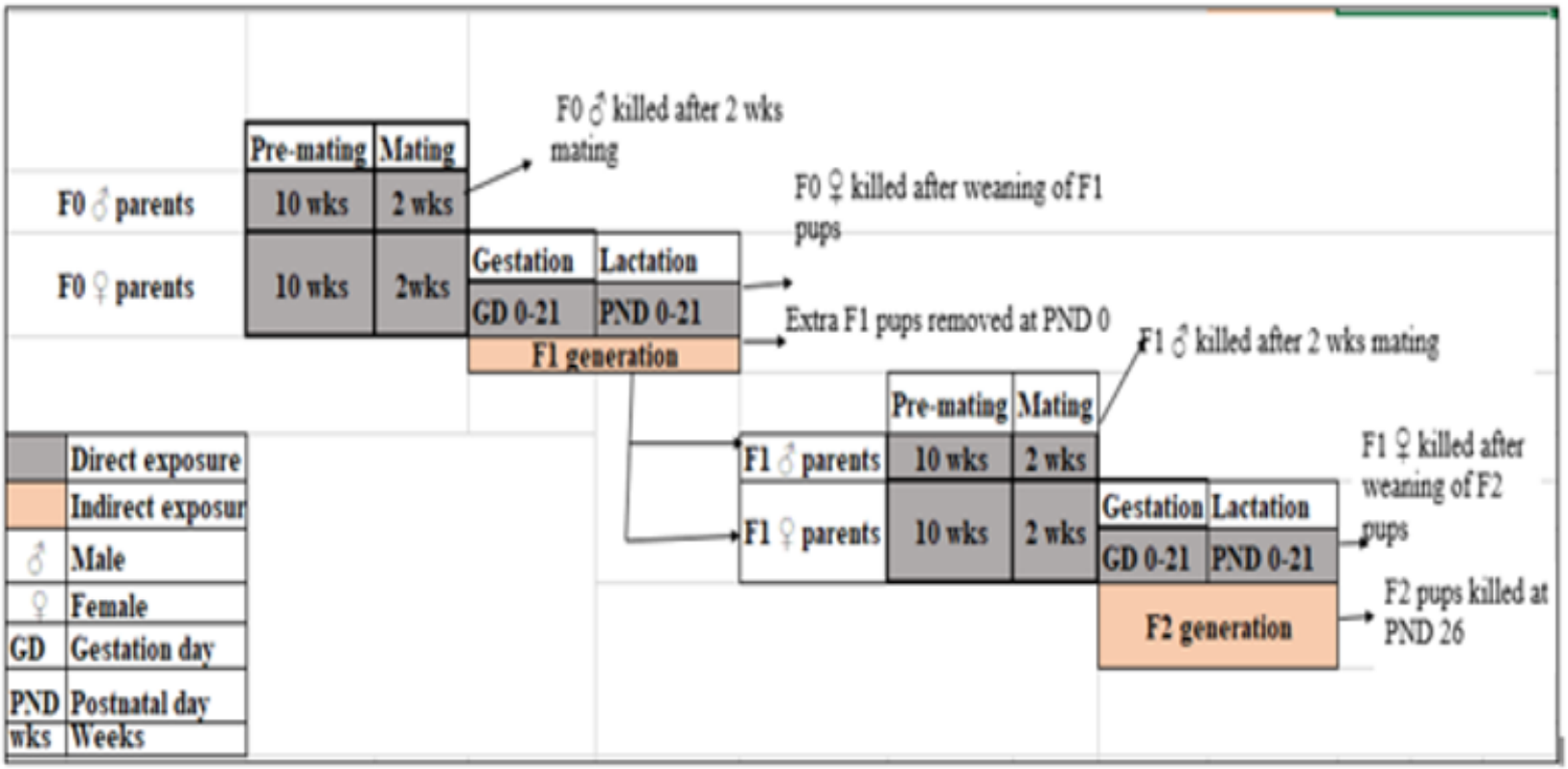


Figure 10: Schematic diagram of study design for two-generation reproductive toxicity showing the period of direct and indirect exposure of animals' and period of necropsy

4.6.6. Reproductive Toxicity Test

4.6.6.1.Reproductive Indices in F0 and F1 Parental Rats

In this study, many reproductive indices were examined. Indicators like mating, fecundity, gestation, live births, viability, and lactation were computed to assess the fertility of the parent rats and their reproductive success. In addition, sex ratio of the offspring was recorded. These indices have been evaluated using the following criteria (Table 5).

Table 5: Reproductive indices used to assess reproductive toxicity

S. No	Reproductive indices	Description
1	Mating/copulation index	(No. of females mated/ no. of females placed with males) x100
2	Fertility index	(No. of females pregnant/no. of females placed with males) x100
3	Fecundity index	(No. of females pregnant/no. of females mated) ×100.
3	Gestation/pregnancy index	(No. of females with live pups/no. of females pregnant) ×100
4	Live birth index	(No. of live offspring/ Number of offspring delivered) × 100
5	Sex ratio	No. of male offspring /Number of female offspring
6	4-day survival/Viability index	(No. of live offspring at lactation day 4/ Number of live offspring delivered) × 100
7	Lactation/ Weaning index	(No. of live offspring at day 21/ Number of live offspring born) × 100
8	Preweaning index	(No. of live offspring born - Number of offspring weaned/ Number of live offspring born) × 100
9	Delivery index	(No. of live pups delivered / no. of implantations) ×100
10	Abortifacient index	(No. of aborted rats/no. of pregnant rats) ×100
11	Total post implantation loss	((No. of implantation sites−no. of live births)/no. of implantation sites) ×100.

4.6.6.2.Estrous Cycle Measurement

The length and normality of the estrous cycle were assessed through daily vaginal smears for F0 and F1 parent rats. The vaginal smear was examined for 2 weeks before and during the mating period until signs of mating was observed. Five rats in each group were randomly selected to examine the estrous cycle. To prepare the smear, rats were placed in a restraining device and vaginal fluid was taken through a plastic pipette filled with 10 µl of normal saline (0.9% Na Cl). The tip of a plastic dropper was carefully inserted into the vagina. The vaginal fluid was spread evenly over the labelled glass slides. The slides were kept at room temperature until they dry. For staining purposes, a few droplets of crystal violet were added to it and kept for one minute. The crystal violet was washed with distilled water and glycerol was added to increase the optical property. It was then covered with a cover slip and examined under a binocular optical microscope [27].

Through microscopic examination of the slides, leukocytes, cornified cells, and nucleated epithelial cells were identified. The following cellular features allowed us to identify these cells. Epithelial cells were rounded and nucleated, cornified cells were amorphous without a nucleus, and the little round cells were leukocytes. The phases of the estrous cycle were calculated based on the percentage of these cells. Proestrus, estrus, metestrus, and diestrus were named the four estrous cycle phases. An estrous phase mostly consisted of non-nucleated cornified cells, but a proestrus phase was distinguished by the predominance of nucleated epithelial cells. The metestrus phase of the estrous cycle was determined by the smear's comparable distribution of leukocytes, cornified cells, and nucleated epithelial cells. Finally, the predominate leukocytes on the smear helped to identify it as being in the diestrus phase. One estrous cycle was thought to last for the entirety of these phases [135, 136].

Each and every F0 and F1 female rat gave birth normally. From GDs 21 to 25, they were examined for indications of parturition three times a day (in the morning, midday, and late afternoon). The number of days from GD one to the end of parturition was used to represent the gestation length. The number of implantation sites for each rat was counted at necropsy [120].

4.6.7. Litter Data

Before weaning, each F1 and F2 pup was checked daily for clinical symptoms and death. The size and sex of each litter were noted on PND 0 for each litter. Every pup in every litter was weighed individually on PND 0, 4, 7, 14, and 21. The anogenital distance (AGD), which is the measured distance between the anus and the external genitalia, is a sexually dimorphic physical marker in humans and rodents that represents the androgenic action during the development of the reproductive system in utero [137]. AGD was assessed in this study on PNDs 0 and 4 for all F1 and F2 pups using a calliper. Viability indices were computed after counting the number of pups that survived in each litter on PNDs 0, 4, and 21. Indicators of development such as the age at which pinnae separate, incisors erupt, hair sprouts, ears open, and eyes open were also assessed.

The F1 and F2 were subjected to a sexual maturation examination. In the selected F1 and F2 rats, sexual maturation was assessed by scoring the day of the vaginal opening in females starting from PND 22 and preputial separation in males from PND 35 onwards [138].

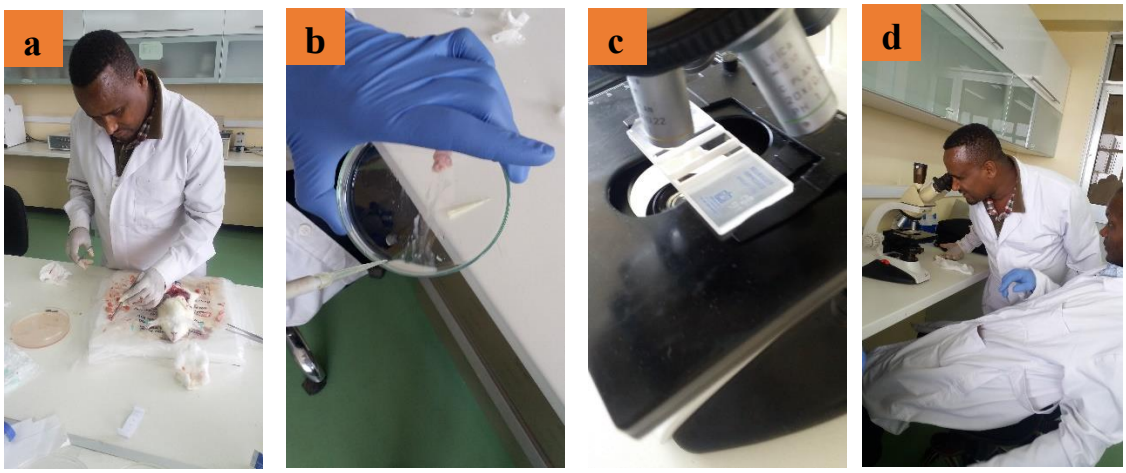
4.6.8. Hormone and Biochemical Analysis

In order to conduct clinical chemistry tests and hormone analysis, 5 ml of blood sample was collected via cardiac puncture from each of the parental rats. The blood was placed in a test tube for an hour and centrifuged for ten minutes at 3500 revolutions per minute in an electrical centrifuge. The serum was then meticulously extracted using a micropipette and stored in a bottle. Finally, an automated clinical chemistry analyzer examined the serum (Cobas 6000 (c501 and e601)) in the EPHI national clinical chemistry reference laboratory accredited by Ethiopian National Accreditation Office with facility accreditation number M0025 to perform medical testing. Then serum electrolytes, liver, and kidney biomarkers were investigated. Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) were analyzed in both male and female rats. Testosterone in male rats and estrogen and progesterone in female rats were also analyzed.

4.6.9. Semen Analysis

At necropsy, semen was collected from the caudal end of the left epididymis, and precautions were taken to avoid exposure to heat. To examine the sperm count, a 0.1ml

sample of semen was diluted with 1% formalin (40%) in a 2 ml volumetric flask, and the semen was diluted with a 1:20 dilution factor. The flask was shaken very well, and a single drop of sperm suspension was placed into a counting chamber, the Neubauer haemocytometer chamber, and kept settled in a humid place for 10 minutes. After the sperm settled on the grid, they were counted in five squares using a 40x objective lens. The total number of sperm cells was calculated as the sperm count in five squares was multiplied by 10^6 to determine the number of sperms per millilitre. The sperm count was done using the following formula. Sperm count = dilution x (count in 5 squares) x 0.05 × 10^6 [139] (Figure 11).



semen collection | semen dilution | sperm suspension placed into haemocytometer chamber | counted in five squares

Figure 10: Procedures for sperm analysis

To examine sperm morphology, the sperm cells were collected from the left epididymis, and the suspension was smeared, dried, fixed with fixative (three volumes of absolute methanol and one volume of glacial acetic acid), and was stained with hematoxylin for 15 minutes, and washed then stained with 1% eosin for 10 minutes, and was washed again and left to dry at room temperature [140, 141].

The sperm motility was evaluated using the standard method as described by Sonmez et al. [142]. The fluid was obtained from caudal epididymis using a pipette and diluted to 2 ml with Tris buffer solution. Then the slide was placed on the phase contrast microscope and an aliquot of this solution was dropped on the slide and percent motility was evaluated visually at a magnification of 400. The sperm motility estimations were made from three

different fields in each sample. The mean of the three estimations was used as the final motility score.

4.6.10. Termination/ Autopsy of parental animals

At the end of the experimentation, all animals were slaughtered with an intraperitoneal injection of pentobarbital (150 mg/kg body weight) [123]. A midline incision on the anterior abdominal wall was made to expose the visceral organs. Then the testes, epididymis, prostate, and seminal glands in male rats and ovaries and uterus in female rats were cleaned and freed from surrounding fat tissues. These organs were macroscopically examined for any structural anomalies or treatment-related changes. The weights of the ovary, uterus, testes, epididymis, and prostate gland were recorded before fixation. The weight of seminal vesicles was measured after fixation. For bilateral organs, values were the average of the two. Relative organ weights were also calculated by considering the animal's weight taken on the day of necropsy [143].

4.6.11. Termination/ Autopsy of Pups

F1 and F2 pups not selected on PND 4 were killed by intraperitoneal injection of pentobarbital and autopsied on that day. The F1 weaning pups that were not selected as F1 parental animals and all F2 weaning were killed by intraperitoneal injection of pentobarbital and autopsied at 26 days of age [143].

4.6.12. Histopathological Examination

In the control and high dose groups of F0 and F1 parent animals, reproductive organs such as testes, epididymis, seminal vesicles, prostate gland, uterus, ovary and vagina were examined by light microscope for any histopathological abnormalities. The sample from each organ was fixed with 10% formalin and further processed following routine tissue processing step [143]. A detailed microscopic examination, for any treatment-related changes, was performed by a senior pathologist using a binocular light microscope. The histological appearance of organs from the treatment groups was compared with the control group. After investigation, illustrative photomicrographs were captured with an automated inbuilt digital microscope camera (Leica EC4, Germany) under a total magnification of 40x and 100x by the pathologist in St. Paulo's Millennium medical college.

4.7.Statistical Analysis

The data were coded, entered, and analysed using Statistical Package for Social Sciences (SPSS) version 24. The data concerning maternal body weight and food consumption, outcomes of pregnancy, reproductive indices, embryonic development, fetal growth, relative weight of the reproductive organs, number of sperm cells, motility of sperm cells, and structure of sperm cells were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey post hoc test for significant differences between groups. To ascertain whether or not the means of the treatment and control groups were significantly different, one-way ANOVA was performed. Dunnett's test was also employed to assess the difference between each treatment group. Dunnett's test was also used to compare each treatment group with the two control groups. The Shapiro-Wilk test of normality was used to determine if the data scores were normally distributed or not. To satisfy the requirement for the assessment of the homogeneity of variance, Levene's test was run on the data prior to performing an ANOVA. Additionally, a chi-square test was run to see if the treatment and control groups differed in terms of the percentage of embryos or fetuses with abnormalities. The Chi-square test was used to assess the presence of an association between the frequency of anomalies and the test substance's use. The findings were presented as figures, tables, and texts and expressed as mean ± standard deviation of the mean (SDM) and percentages. Results were considered statistically significant if the *p-value* was less than 0.05.

4.8. Ethical Approval

All protocols were approved by the Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University (Form AAUMF 03-008, Elements Reviewed AAUMF 01-008) and the Department Graduate Committee (DGC) of the Anatomy Department (Protocol number 084/18/ANAT). The treatment of the animals complied with the International Guidelines for the Care and Use of Laboratory Animals [115, 144]. The experimental animals were cared ad managed following the OECD guidelines prepared for the caring and managing laboratory animals [145] and they were humanely kept in the biomedical research laboratory of EPHI. The rats were not exposed to somewhat needless, painful, or frightening manipulations. The extract was provided by an expert and an all-out effort was applied to prevent contamination and introduction of pathogens to the animals.

To avoid pain and suffering, the rats were anesthetized with pentobarbital. Finally, sacrificed rats were disposed of humanely following the laboratory standards of EPHI.

4.9.Variables

4.9.1. Dependent Variables

- Food intake
- Craniofacial development
- Development of the limbs
- Development of Vertebral column
- Tail development
- Development of External genitalia
- Number of the ossification center
- Degree of ossification
- Yolk sac circulation
- Allantois development
- Development of the nervous, circulatory, auditory, visual, olfactory, and development of craniofacial and skeletal systems
- Number of somites
- Crown-rump length
- Weight of animals
- Weight of organs

- Levels of reproductive hormones such as follicle-stimulating hormone, luteinizing hormone, Testosterone, estrogen, progesterone
- Sperm count
- Sperm motility
- Sperm morphology
- Estrous cycle length
- Anogenital distance of puppies
- Number of puppies, sex ratio of puppies
- Sexual maturation: separation prepuce in male and opening of vaginal in female
- Number of live births
- Fertility, Fecundity, Mating, Gestation, Viability, as well as lactation parameters
- Gestation time
- Post implantation loss
- Developmental landmarks
- Gross and histology of reproductive organs such as ovaries, vagina and uterus in female and epididymis, testes, prostate gland, and seminal vesicles in male
- Serum biochemical profiles

4.9.2. Independent Variables

- The study's independent variables were the two control groups, as well as the chosen doses of *M. stenopetala* leaves extracts such as 250 mg/kg, 500 mg/kg, and 1000 mg/kg body weight.

4.10. Definition of Terms

- **Reproductive toxicity** - The occurrence of biologically adverse effects on the reproductive systems of females or males that may result from exposure to environmental agents.
- **Developmental toxicity** - The occurrence of harmful effects of a substance on the developing organism that may cause due to exposure to the substance before conception, during pregnancy, or postnatally until sexual maturity.
- **Day 12 Experiment** - The experimental design that is used to identify any growth and developmental delays and abnormalities on 12-day-old rat embryos that would not have been obvious in the fetuses that would have been born soon because of potential compensatory growth and development.
- **Day 20 Experiment** - The experimental design that is used to assess any growth and developmental delays and abnormalities on 20 days old fetuses.
- **Two-generation reproductive toxicity Study –** Studies that look for adverse effects of a substance on the healthy and presentation of the male and female parental (F0) and first generation (F1 generation) rats. In addition, the study intended to evaluate the effects on the developmental status and growth of F1 generation as well as second generation (F2 generation) offspring.
- **F0 pare**ntal animals – Parental rats that are used to produce F1 generation of animals' and that are used for studying parental reproductive toxicity study of a substance.
- **F1 pare**ntal animals **–** Parental rats that are used to produce F2 generation of animals' and that are used for studying F1 reproductive toxicity study of a substance.
- **Fertility** - The capacity to conceive or induce conception.
- **Fecundity** - The ability to produce offspring within a given period.
- **Fertile** - A level of fertility that is within or exceeds the normal range for that species.
- **Infertile** - Lacking fertility for a specified period. The infertile condition may be temporary; permanent infertility is termed sterility.
- **Implantation (nidation)** - the process where the blastocyst imbedded to the endometrium of the uterus.
- **Embryo** - the developing organism from fertilization of an egg after the long axis appears and till all main structures are existing.

- **Embryotoxicity-** toxicity to the embryos that harmful to developmental status, growth, and/or viability of an embryos and to their normal structure
- **Fetus-** the intrauterine offspring during post-embryonic period.
- **Fetotoxicity –** toxicity to the fetuses that detrimental to developmental status, growth, and/or viability of a fetus and to their normal structure.
- **Abortion-** the expulsion the concepts or the embryo or of a nonviable fetus from the uterus.
- **Resorption -** when implanted conceptus in the uterus died or has been resorbed. if there is no evidence of recognizable embryo/fetus in the implantation it is called early resorption. If there is dead embryo or fetus with external degenerative changes, it is called late resorption.
- **Litter**- multiple newborn rats at once.
- **Crown Ramp Length (CRL)-** is the distance from the buttock apices to the vertex of the cranium.
- **Apoptosis**- is a kind of cell death characterized by nuclear DNA fragmentation, in which cells are eliminated by a predetermined series of events without the release of toxic compounds into the surrounding environment.
- **Necrosis-** is a kind of cell damage that causes the early death of cells in live tissue by autolysis.
- **Cytolysis-** is the pathological destruction of a cell owing to the breaking of the cell membrane brought on by osmosis.
- **Estrous cycle-** a sequence of physiological events in sexual and other organs in female mammals, ranging from one period of heat to the next, go with by behavioral variations indicative of curiosity in copulating.
- **Normal estrous cycle-** the four phases of estrous cycle lasts 4 to 5 days. These phases are namely proestrus, estrus, metestrus and diestrus.
- **Abnormal estrous cycle-** when progression of the regular female reproductive cycle failed in non-primate animals.
- **Sexual maturation in rats-** postnatal age (34-39 days) when opening of the vagina in female and separation of the prepuce (38-42 days) in male rats.
- **Abnormality of sperm structure**- sperm cells with abnormal head or tail such as a presence of large head or misshapen head or double or crooked tail.

4.11. Communication of the Findings

The findings of the current investigation were reported to the Wollo University Addis Ababa University, Anatomy Department, and EPHI, particularly to the TMDRD. The findings were also published in indexed scientific journals and disseminated globally to the scientific community.

CHAPTER FIVE

5. RESULTS

5.1.Results of the Teratogenicity study

5.1.1. Day 12 Experiment

5.1.1.1. Maternal food intake and weight measurements

The maternal daily food intake and weight gain of the pregnant rats are briefly summarized in Table 6. The daily food intake of the pregnant rats was assessed both before (days 1–5) and during (days 6–12) the treatment periods. During the pre-treatment period, significant difference was not observed in the daily food intake between the treated and control groups. However, from days 6 through 12, the amount of food consumed per day tended to decline. Both the high dose treated animals (17.40±1.17 g) and the pair-fed control animals (19.20±5.59 g) experienced weight gain. It was slightly decreased in the high dose treated animals, but the finding was not reach statistical level (Table 6).

Table 6: Maternal daily food consumption and weight gain following treatment with *M. stenopetala* leaf extracts

Groups (n=10 for each group)	**Food consumption and body weight (g)**		
	FI/day/dam		**BW/dam**
	Day 1 to 5	**Day 6 to 12**	**Day 6 to 12**
Group I	182.62±2.73	177.36±14.95	18.30±4.76
Group II	184.25±14.19	174.35±7.68	18.40±2.46
Group III	182.06±13.52	168.63±5.57	17.40±1.17
Group IV	182.98±13.48	173.45±9.40	19.20±5.59
Group V	178.16±12.96	201.76±20.33	23.90±5.11

- mean ± SDM; One-way ANOVA, n: number of animals
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.1.1.2.Pregnancy Outcomes

To evaluate the pregnancy outcomes, the implantation sites, resorption sites, and the number embryos were counted. In 1000 mg/kg treated animals, the number of resorption sites was significantly increased as compared to the low dose treated and the pair-fed control groups. In addition, the number of embryos was significantly decreased in 1000 mg/kg treated animals than the pair-fed group. The mean number of embryos in the high dose treated and pair-fed control groups was 103 and 114, respectively. Furthermore, fewer implantation sites were observed in animals treated the higher dose of the extract than in the lower and middle doses treated, and the pair-fed control groups, yet not reach the statistical level (Table 7 and Figure 12).

Table 7: Pregnancy outcomes following treatment of pregnant rats with *M. stenopetala* leaf extracts

Groups (n=10 for each group)	**Number of implantation sites/litter**	**Number of resorption sites/litter**	**Number of embryos/groups**
Group I	10.70 ± 0.95	0.4 ± 0.70	110
Group II	11.40 ± 1.08	0.6 ± 1.08	108
Group III	10.50 ± 0.71	1.2 ± 1.03*	103**
Group IV	11.10 ± 0.74	0.3 ± 0.48	114
Group V	11.00 ± 0.89	0.2 ± 0.42	108

- The findings are stated as the mean ± SDM.
- ∗Significantly different from 250 mg/kg and the pair-fed groups
- **Significantly different from pair-fed control animals (*p-value* < 0.05); one-way ANOVA; n: number of pregnant rats.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.1.1.3.Evaluation of Embryonic growth

The degree of embryonic growth in the current study was assessed by using the morphological scores, counting somite numbers, measuring CRL and yolk sac diameter. The CRL was reduced significantly in the 1000 mg/kg treated animals than in the pair-fed control group. The CRL of the embryo measured 5.35 ± 0.99 mm and 4.28± 0.89 mm,

respectively, in the pair-fed as well as 1000 mg/kg treated animals. The somite number as well as diameter of the yolk sac were also significantly lower in the middle and high doses treatment animals. The morphological scores were significantly decreased in 1000 mg/kg treated than the pair-fed control animals that were 43.3±2.45 and 45.7±2.16, respectively (*p value* < 0.05) (Table 8).

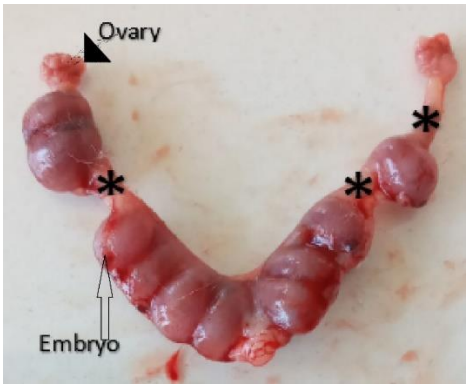


Figure 11: The 12 days old gravid rat uterus shows embryonic resorption sites taken from 1000 mg/kg body weight treated animals. *Embryonic resorption sites

Table 8: Embryonic growth evaluation following administration of *M stenopetala* leaf extracts to pregnant rats

Groups	**Embryonic growth Variables**			
	CRL of embryo/litter(mm)	**No. of somites/litter**	**Yolk sac diameter/litter (mm)**	**Morphological score/litter**
G-I: (n=110)	4.69±0.62	29.60±2.41	4.5±0.53	44.60±1.35
G-II: (n=108)	4.59±0.98	28.20±1.93*	4.3±0.71*	44.30±1.34
G-III: (n=103)	4.28±0.89*	27.80±1.32*	3.9±0.43*	43.3±2.45*
G-IV: (n=114)	5.35±0.99	31.30±1.64	4.6±0.23	45.7±2.16
G-V: (n=108)	5.83±1.13	32.40±3.50	4.6±0.82	46.1±1.86

- The findings are expressed as mean± SDM;
- *significance difference from pair-fed control group; (*p-value* < 0.05) one-way ANOVA;
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control, n: number of embryos; mm: millimeter.

5.1.1.4.The Developmental Status of the Body System in rat Embryos

In rat embryos taken from the high dose and middle dose treated animals, the morphological scores for the otic system was decreased. In addition, the morphological scores for the development of the mandibular process, brachial bars, optic, and olfactory systems, were also decreased in the high dose treated animals however, not reach to the statistical level (Table 9).

Tables 10–12 in the current study provide information on developmental delays in the body systems of rat embryos that aged 12-days. The proportion of developmental retardation of the yolk sac circulation was higher in 1000 mg/kg treated animals than in 250 mg/kg, 500 mg/kg treated, and the pair-fed control animals, yet the result was not statistically significant.

In this investigation, the percentage of retarded somite scores in the pair-fed control and 1000 mg/kg treated animals was 2.6% and 10.7%, respectively. Furthermore, the percentages of retarded otic system development in 1000 mg/kg treated and the pair-fed control animals were 2.6% and 9.7%, respectively. Developmental retardation of the somites and otic system was much more pronounced in 500 mg/kg and 1000 mg/kg treated animals that 250 mg/kg treated and the pair-fed control animals. The developmental status of the primordial nervous system (caudal neuropore, forebrain vesicle, midbrain vesicle, hindbrain vesicle), heart, allantois, optic system, and the olfactory system, however, did not differ much from one another. The development of the craniofacial and musculoskeletal systems, including the forelimbs, hindlimbs, brachial bars, maxillary process, mandibular process, and degree of flexion of the embryos, did not change significantly between the treatment and control animals. Figure 13 illustrates the primordia of the embryo's bodily systems in terms of their developmental stages.

Table 9*: In vivo* developmental status of rat embryos following treatment of pregnant rats with *M. stenopetala* leaf extracts

Morphological end points	**Treatment groups**			**Control groups**	
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of embryos/groups	110	108	103	114	108
Yolk sac circulation	3.6±0.49	3.5±0.67	3.3±0.00	3.6±0.52	4.1±0.52
Allantois development	3.0±0.00	2.8±0.42	2.8±0.42	3.0±0.00	3.0±0.00
Flexion	2.82±0.00	3.00±0.00	3.00±0.00	2.63±0.52	2.65±0.52
Heart	3.71±0.52	3.44±0.42	3.43±0.42	3.82±0.52	3.74±0.42
Caudal neural tube	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Hindbrain	3.54±0.50	3.64±0.45	3.53±0.42	3.63±0.52	3.56±0.42
Mid brain	3.62±0.23	3.70±0.24	3.63±0.43	3.66±0.52	3.64±0.42
Forebrain	3.70±0.33	3.64±0.42	3.60±0.32	3.78±0.73	3.76±0.46
Otic system	3.47±0.43	3.21±0.26*	3.13±0.42*	3.62±0.52	3.63±0.52
Optic system	2.45±0.52	2.40±0.42	2.03±0.52	2.52±0.42	2.58±0.42
Olfactory system	0.51±0.52	0.43±0.42	0.34±0.00	0.73±0.52	0.70±0.42
Branchial bars	3.62±0.34	3.43±0.54	3.13±0.42	3.81±0.52	3.84±0.52
Maxillary process	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00
Mandibular process	0.63±0.00	0.44±0.00	0.30±0.00	0.73±0.00	0.75±0.00
Fore limb	2.00±0.00	2.23±0.42	2.00±0.00	2.21±0.42	2.23±0.42
Hind limb	2.20±0.42	2.22±0.42	2.00±0.00	2.21±0.42	2.23±0.42

- Data are presented as mean ± SDM
- *Significantly vary from pair-fed control group
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- For all *p values* <0.05; one-way ANOVA.

Table 10: Embryonic circulatory system development following administration of pregnant rats with *M. stenopetala* leaf extracts

Groups	**Percent of retarded development**		
	Heart	**Yolk sac circulation**	**Allantois**
Group I (n=110)	0	1.8	0
Group II (n=108)	0	2	0
Group III (n=103)	1.9	4.9	1
Group IV (n=114)	0	2.6	0
Group V (n=108)	0	0	0

- The results are stated as the percentage (%) of retarded development.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- n: number of embryos.

Table 11: Embryonic nervous system and sense organ development after administration of 70% ethanol extracts of *M. stenopetala* leaf to pregnant rats

Groups	**Percent of retarded development**						
	FB	**MB**	**HB**	**CNP**	**Optic system**	**Olfactory system**	**Otic system**
G-I: (n=110)	0.9	0	0	0	0	0.9	2.7
G-II:(n=108)	1.9	0	0	0	0	0.9	7.4*
G-III:(n=103)	3.9	2.9	0	0	2.9	2.9	9.7*
G-IV: (n=114)	1.8	0.9	0	0	0.9	0.9	2.6
G-V: (n=108)	0.9	0	0	0	0	0	0.9

- The results are stated as a percentage (%) of retarded development.
- *Significantly different from the low dose treated and pair-fed control animals (*p-value* < 0.05) (Chi-Square test).
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- n: number of embryos;
- FB-forebrain, MB-midbrain, HB-hindbrain, CNP-caudal neuropore.

Table 12: Development of embryonic musculoskeletal system following administration of pregnant rats with *M. stenopetala* leaf extracts

Groups	Percent of retarded development						
	FL	HL	BB	MXP	MP	F	SS
Group I (n=110)	0	0	0	0	0	0	4.6
Group II (n=108)	1.9	0.9	0	1.9	0	0	8.3*
Group III (n=103)	0.9	2.9	0	2.9	0.9	0	10.7*
Group IV (n=114)	0.9	0.9	0	0.9	0	0	2.6
Group V (n=108)	0	0	0	0	0	0	1.9

- Data are presented as a percentage (%) of retarded development.
- *significantly different from pair-fed control group (Chi-Square) (*p value* < 0.05).
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- n: number of embryos; FL: forelimb; HL: hindlimb; BB: brachial bars; MXP: maxillary process; MP: mandibular process; F: flexion; SS: somite score.

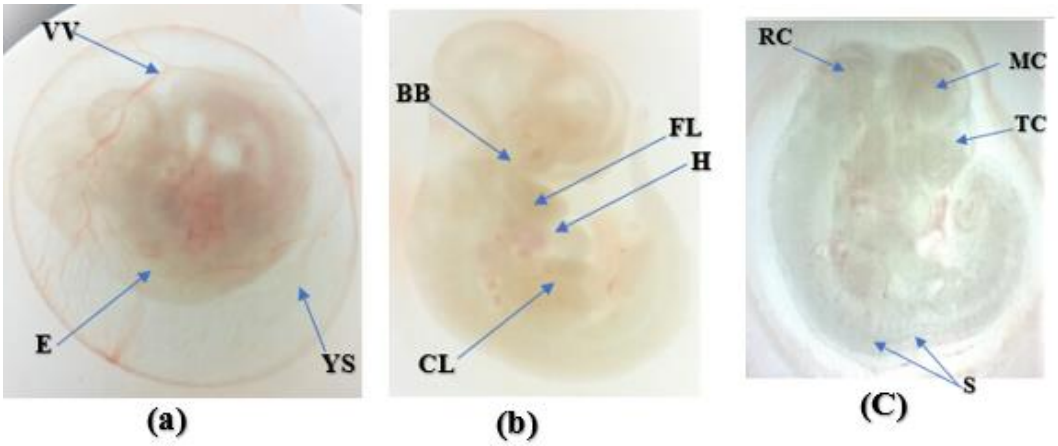


Figure 12: Twelve-days-old rat embryos show the primordia of organs. (a) An embryo (E) confined in the whole yolk sac (YS) surrounded by vitelline vasculature (VV); (b) embryo exposed from membrane, revealed the heart (H), branchial arches (BB), buds for forelimb development (FL), and buds for caudal limb development (CL); (c), showing telencephalon (TC), mesencephalon (MC), rhombencephalon (RC)and somites (S)

5.1.2. Day 20 Experiment

5.1.2.1. Maternal Food Intake and Body Weight Gain

From gestation day 1 to 5 in the current investigation, there was significant difference in the daily food consumption between the experimental and control groups. Even though it was not statistically significant, maternal daily food intake was decreased in all the treated groups during treatment (days 6 to 12) and post treatment (days 13 to 20) periods. Figure 14 depicts the pregnant rats' daily food intake during the whole gestation period.

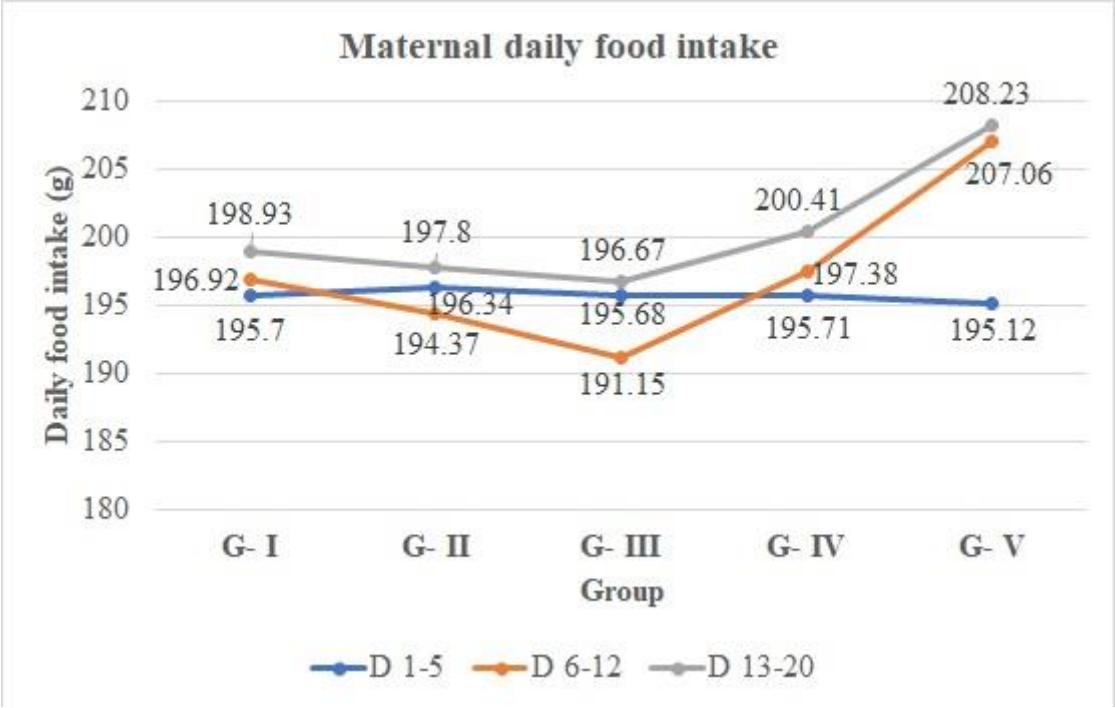


Figure 13: Mean maternal daily food intake (g/day) of pregnant rats treated with *M. stenopetala* leaf extracts in the day 20 experiment

During the pre-treatment period that was from the days 1 to 5 of the pregnancy, no variation in the maternal weight gain between the experimental and control groups. Although it was not statistically significant, the body weight was tended to decline in 1000 mg/kg treated animals during (gestation day 6 to 12) and after (gestation days 13 to 20) treatment periods (Table 13).

Table 13: Maternal body weight of pregnant rats treated with *M. stenopetala* leaf extracts

Groups	Body weight gain of the animals (in gram)		
	Day 1-5	**Day 6-12**	**Day 13-20**
Group I	12.2±1.55	17.4±3.81	62.8±4.37
Group II	11.4±2.12	17.1±2.69	61.5±3.14
Group III	12.1±1.60	16.3±4.00	58.9±10.00
Group IV	11.8±3.16	18.9±2.51	65.1±6.28
Group V	11.7±1.70	19.0±2.11	68.7±4.95

- Data are presented as mean ±SDM; one-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.1.2.2.Pregnancy Outcomes

The outcomes of the rat pregnancies in this investigation are presented in Table 14 and illustrated in Figure 15. When evaluating the pregnancy outcomes, the quantity of implantation and resorption sites, as well as the proportion of dead and living fetuses were all taken into consideration.

All animals in the treatment groups had fewer implantation sites and live fetuses than the pair-fed control group, yet, these were not statistically significant. The fetal resorptions were, however, significantly increased in 1000 mg/kg treated than 250 mg/kg, 500 mg/kg and the pair-fed control groups, ($p < 0.05$).

5.1.2.3.Evaluation of Fetal Growth Indices

In the present study, the fetal growth was assessed by weighting the fetal membrane, live fetuses, placenta, and measuring of fetal CRL. The placental and fetal weights were significantly decreased in 1000 mg/kg treated animals. In addition, the CRL of the fetus was also significantly decreased in 1000 mg/kg treated animals. The CRL of fetuses 1000 mg/kg treated and pair-fed control groups was 4.9±0.47 cm and 5.6±0.36 cm, respectively (Table 15).

Table 14: Pregnancy outcomes following administration of *M. stenopetala* leaf extracts to pregnant rats

Variables	**Treatment groups**			**Control groups**	
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of fetuses	109	106	105	112	113
No. of implantation sites/litter	11.3±0.48	11.0±1.89	10.7±0.82	11.5±1.08	11.3±1. 06
No. of resorption sites/litter	0.4±0.70	0.4±0.48	0.6±0.42*	0.4±0.48	0.4±0.51
No. of live fetuses/litter	10.9±0.74	10.6±1.58	10.1±0.53	11.2±1.14	11.3±1.06
No. of dead fetuses/litter	0	0	0	0	0
Weight of gravid uterus	68.7±7.64	68.3±10.63	69.3±4.75	67.0±10.17	65.4± .39
No. of male fetuses/dam	5.8±1.14	5.3±0.82	4.6±1.08	4.9±1.29	6.0 ±0.74
No. of female fetuses/dam	4.9±0.88	5.3±1.16	5.9±0.74	6.1±1.45	5.2±1.14

- Data are presented as mean ±SDM
- *Significantly different from 250 mg/kg, 500 mg/kg treated and pair-fed control groups: One-way ANOVA; (P value < 0.05)
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- n: number of pregnant rats.

Figure 14: Fetal resorption (*) and implantation sites (I) in the gravid uterus of a rat treated with *M. stenopetala* leaf extracts (a) from pair-fed control, (b) from middle dose treated, (c) from the high dose treated groups of animals

Table 15: Fetal growth following treatment with 70% ethanol extracts of *M. stenopetala* leaf

Groups	**Fetal growth indices**			
	WP/fetus (g)	**WFM (g)**	**CRL/fetus (cm)**	**FW/fetus(g)**
Group I	0.66±0.03	0.3±0.03	5.1±0.54	4.7±1.33
Group II	0.67±0.10	0.3±0.09	5.0±0.46	4.7±0.80
Group III	0.61±0.04*	0.2±0.03	4.9±0.47*	4.3±0.82*
Group IV	0.71±0.03	0.2±0.03	5.6±0.36	5.3±0.91
Group V	0.72±0.05	0.2±0.06	5.6±0.71	5.4±0.44

- Data are summarized mean± SDM,
- *Significantly different from pair-fed control group
- *p- value* < 0.05; one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- WP: placental weight; WFM: weight of fetal membrane; CRL: crown ramp length; FW: fetal weight.

5.1.2.4.External morphological anomalies

The near-term rat fetuses in both the treatment and control animals did not display any externally apparent defects, as shown in Figure 16.

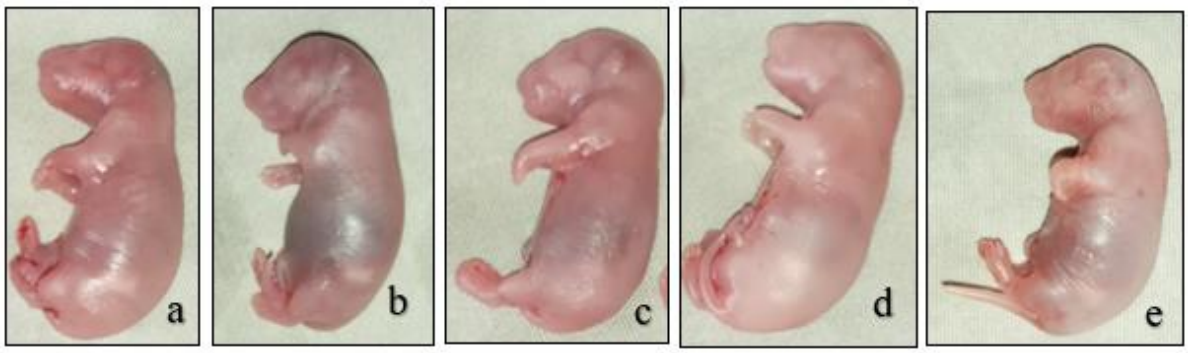


Figure 15: Live fetuses from the three-treatment and the two control groups: a: from low dose treated; b: from middle dose treated; c: from high dose treated; d: from pair-fed control and e: from *ad libitum* control groups

5.1.2.5.Visceral morphological anomalies

In the current study, the visceral organs appeared to be in normal condition all experimental and control groups. The eyes, ventricles, nasal cavity, palate, oral cavity, thyroid, thymus, trachea, and esophagus did not exhibit any developmental abnormalities. In addition, there were no detectable defects in the development of the heart, lungs, diaphragm, abdominal visceral organs, or external genitalia in either the treatment or control groups (Table 16 and Figure 17).

Table 16: Percentage of organ malformations in the fetal soft tissue following exposure of pregnant rats to *M. stenopetala* leaf extracts

Groups	**Percent of fetuses with malformed organ**								
	Hc	**E**	**Cp**	**H**	**L**	**K**	**FL**	**HL**	**Ti**
Group I (n=80)	0	0	0	0	0	0	0	0	0
Group II (n=80)	0	0	0	0	0	0	0	0	0
Group III (n=80)	0	0	0	0	0	0	0	0	0
Group IV (n=80)	0	0	0	0	0	0	0	0	0
Group V (n=80)	0	0	0	0	0	0	0	0	0

- Data are summarized as percentage of fetuses with malformed organs (chi-square).
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- Hc: hydrocephalus; E: eyes; Cp: cleft palate; H: heart; L: liver; K: kidneys, FL: fore limbs; HL: hind limbs, Ti: tail; n= number of fetuses examined.

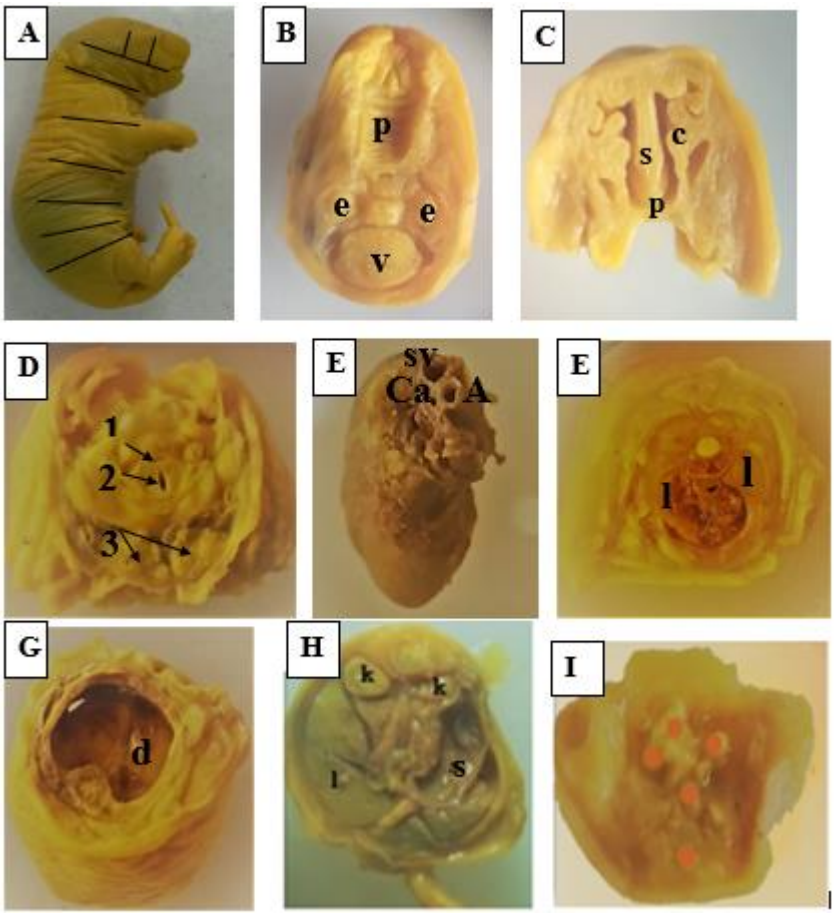


Figure 16: Specimen fixed in Bouin's solution for visceral organ evaluation taken from 1000 mg/kg treated group. (A) Un-sectioned fetal specimen showing sites of sections; (B) transverse section showing the palate (p), showing eye ball (e) and showing brain ventricle (v); (C) transverse section of nasal cavity display nasal septum (s), showing nasal conchae (c), and the palate (p); (D) transverse section on the neck showing 1-esophagus, 2-trachea, 3-thyroid; (E) showing gross feature of the heart with the great vessels like superior vena cava (SV), aorta (A), coronary artery (ca) (F) transverse section on the chest display interventricular septum (s) and lungs (l) (G) complete diaphragm, (H) transverse section on the abdomen showing visceral organs including the liver (l) kidney (k), stomach (s); (I) section showing pelvic visceral organs (doted)

5.1.2.6.Skeletal Malformations

Tables 17 and 18 as well as Figure 18 provide a summary of the results of the skeletal assessments. According to the observations, neither the treatment group nor the control group had any skeletal deformities in the skull bones, thoracic vertebrae, ribs, or hyoid bone. The sternum, sacro-caudal vertebrae, metacarpus, metatarsus, forelimb phalanges,

and hindlimb phalanges all showed changes in the ossification centres among the treated and control groups; yet not reach to the statistical level. However, in 1000 mg/kg treated animals, a statistically greater proportion (40.7%) of rat fetuses lacked proximal hindlimb phalanges when compared to 250 mg/kg treated and the pair-fed control groups.

Table 17: Evaluation of skeletal malformations on 20 days old rat fetuses after exposure of the pregnant rats to *M. stenopetala* leaf extracts

Groups	**Percent of skeletal malformations**						
	Hyoid*	**Sternum***	**Rs****	**CV*!**	**TV****	**LV*!**	**SCV*****
Group I ((n=27)	0	11.1	0	0	0	0	7.4
Group II (n=27)	0	14.8	0	0	0	0	11.1
Group III (n=27)	0	22.2	0	0	0	0	18.5
Group IV (n=27)	0	11.1	0	0	0	0	7.4
Group V (n=27)	0	7.4	0	0	0	0	7.4

- Data are presented as percentage of skeletal malformations (chi-square test)
- *sternebrae with less than 4 ossification centers and hyoid bone not showing sign of ossification
- **Thoracic vertebrae, with less than 13 ossification centers and with less than 13 ribs
- ***Caudal vertebrae with less than 4 ossification centers.
- *! Cervical vertebrae with less than 7 ossification centers and lumbar vertebrae with less than 5 ossification centers.
- CV: Cervical vertebrae; TV: Thoracic vertebrae; LV: Lumbar vertebrae; SCV: Sacro-caudal vertebrae.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

Table 18: Skeletal (limb bones) malformations in 20-days old rat fetuses from pregnant rats exposed to *M. stenopetala* leaf extracts

Groups	**Percent of skeletal malformations of limb bones**			
	Metacarpus$^{*!}$	**Forelimb phalanges**$^{*+}$	**Metatarsal**$^{*!}$	**Hindlimb phalanges**$^{*+}$
G-I: ((n=27)	7.4	18.5	0	18.5
G-II (n=27)	11.1	22.2	7.4	33.3
G-III (n=27)	14.8	25.9	11.1	40.7*
G-IV (n=27)	11.1	11.1	0	18.5
G-V (n=27)	7.4	11.1	0	14.8

- The findings are summarized as percentage of skeletal malformations (chi-square test)
- $^{*!}$ Presence of ≤3 metacarpus and metatarsus.
- $^{*+}$Absent proximal phalanges.
- *Significantly different from 250 mg/kg treated and pair-fed control groups (*P value*<0.05).

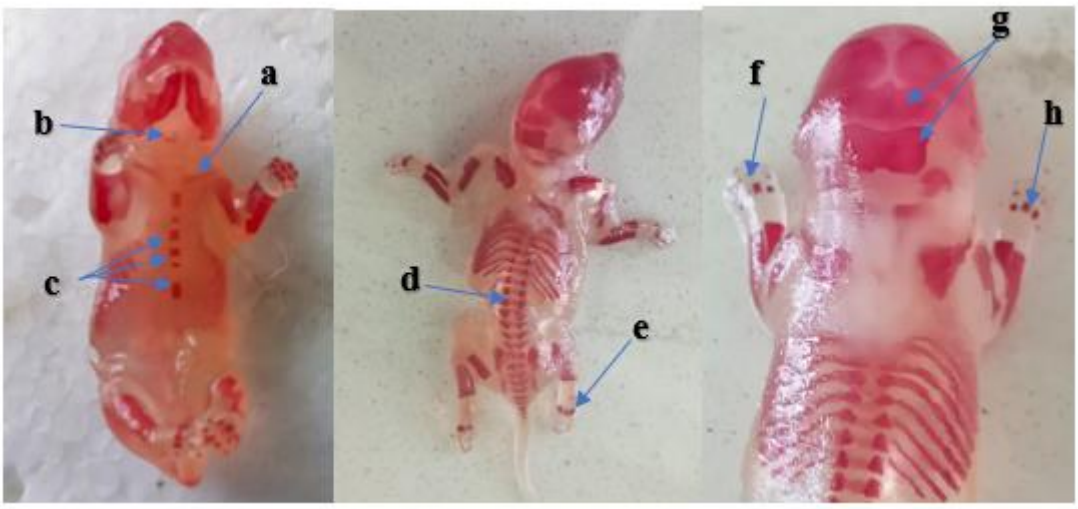


Figure 17 : Specimen taken from 20-day-old rat fetuses and stained using Alizarin red and showing ossification centers. a: clavicle, b: hyoid, c: sternum, d: vertebrae, e: metatarsals, f: forelimb phalanges, g: supra-occipital and interparietal, h: metacarpals

5.1.3. Effects on the Placenta of 20-Day-Old Rat Fetuses

5.1.3.1.Gross Examination of Placenta

The placentae were inspected in the current investigation to look for any gross or microscopic abnormalities. There were no discernible variations in the size, colour, or overall appearance of the placentae between the treatment and control groups, as illustrated in Figure 19.

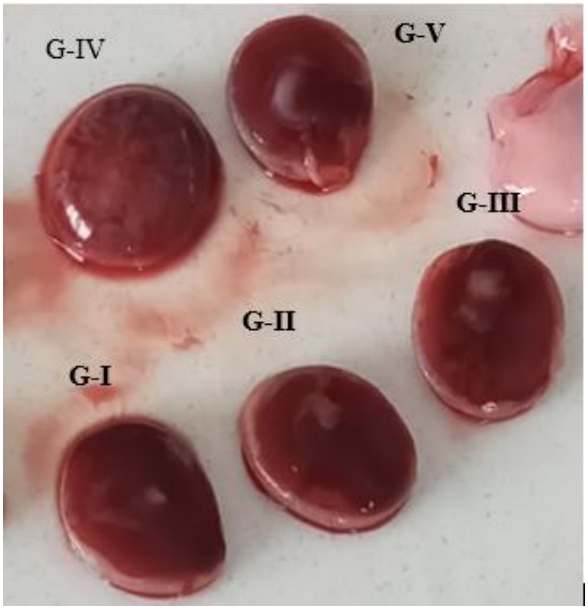


Figure 18: Sample of placenta taken from each group; G-I: from low dose treated; G-II: from middle dose treated; G-III: high dose treated; G-IV: pair-fed control and G-V: *ad libitum* control groups

5.1.3.2.Placental Histopathology Evaluation

Apart from the normal appearance of the gross structure of the placentae, however, light microscopic examinations of the placentae's decidual basalis, trophoblastic, and labyrinthine zones indicated structural alterations (Table 19 and Figure 21). The trophoblastic and labyrinthine zones were filled with hematoma in the placenta of high-dose treated animals (Figure 21b). Like this, all treatment groups' placentae displayed capillary dilatation (Figure 21c). Additionally, every treatment group's placenta displayed decidual necrosis (Figure 21d). Similarly, signs of decidual cytolysis and decidual apoptosis were observed in 500 mg/kg and 1000 mg/kg treated animals (Figure 21e). In addition, a significantly higher trophoblast proliferation was observed in the high-dose

treated animals (Figure 21a) than those in the pair-fed control animals (Figures 20a & b). However, there were no alterations in the glycogen cells.

Table 19: Percentage of placental abnormalities following exposure of pregnant rats to *M. stenopetala* leaf extracts

Placental Abnormality (%)	**Group of Animals**				
	Group I (n=30)	**Group II (n=30)**	**Group III (n=30)**	**Group IV (n=30)**	**Group V (n=30)**
Decidual degeneration	3.3	6.7	10	0	0
Decidual Apoptosis	0	3.3	13.3	3.3	0
Intervillous space thrombosis (hematoma)	0	0	3.3	0	0
Decidual Hypoplasia & Atrophy	0	0	6.7	0	0
Decidual Cytolysis	0	6.7	23.3	0	0
Capillary dilatation	3.3	6.7	13.3	0	0
Trophoblast proliferation	10	23.3	33.3*	16.7	13.3

- The results are stated as the percentage of placental abnormalities, Chi-square.
- *significantly different from *ad libitum* control group (*P value* < 0.05).
- n-number of placentae examined;
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

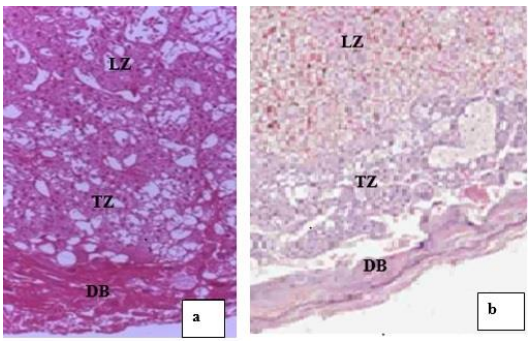


Figure 19: Photographs of the placenta from the pair-fed control (a) and *ad libitum* (b) control animals showing normal structural architecture: decidual basalis (DB); trophoblastic zone (TZ); and labyrinth zone (LZ); E&H stain, 40x magnification.

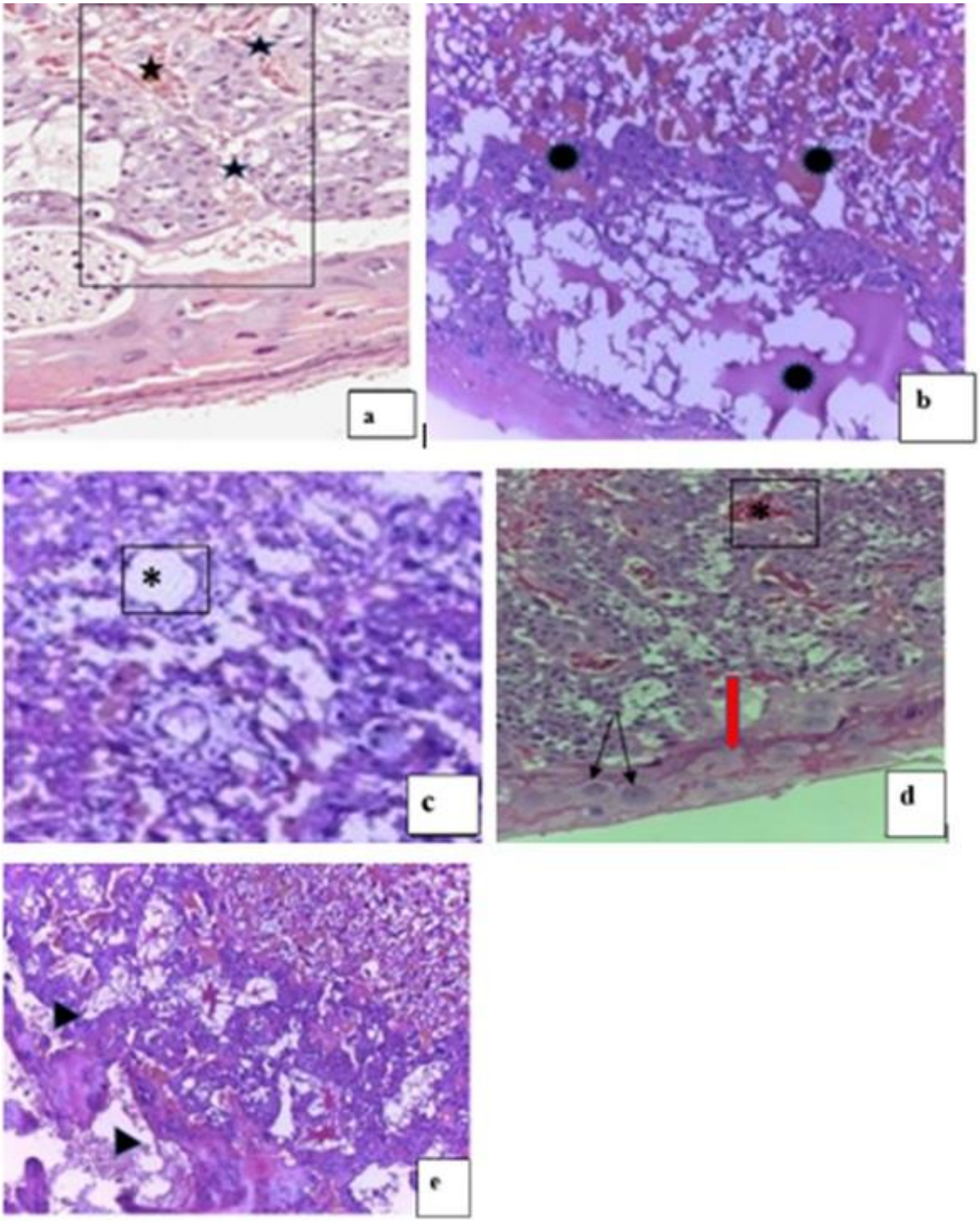


Figure 20: Photographs of the placenta taken from 1000 mg/kg/day treated animals showing (a) proliferation of the trophoblast (★); (b) hematoma in the trophoblastic and labyrinth zones (✹); (c) dilatation of capillary (*); (d) apoptosis in the decidual zone (red arrow), cytolysis in the decidual zone (black arrows); (e) cellular necrosis I the decidual zone (head arrow); E and H stain, a & b using 100x and c, d & e using 40x magnification.

5.2. Two-generation Reproductive Toxicity of a 70% Ethanol Extracts of *M. stenopetala* leaf in Wistar Albion Rats

5.2.1. Clinical observations during premating, mating, gestation, and lactation periods

All F0 and F1 male and female parental rats were survived and no deaths during the premating and mating periods. F0 and F1 Female rats were also survived and no deaths during the gestation and lactation periods. In general, neither of the two generations' treatment groups showed treatment-related clinical toxicity symptoms or behavioral changes.

5.2.2. Food Consumption of F0 and F1 Parental Rats

5.2.2.1. Food Consumption During Premating and Mating Periods

Throughout the dosing period, the food intake of F0 and F1 male and female parental rats was monitored weekly. Food consumption was decreased in 1000 mg/kg treated animals during the premating and mating periods in both F0 and F1 parental animals. However, it was not statistically significant (Tables 20 and 21).

Table 20: Food intake of F0 male and female parental animals during premating and mating periods following treatment with *M. stenopetala* leaf extracts

Groups	**Premating**									**Mating**		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
F0 male rats												
G-I	17.48±0.91	17.66±0.77	17.75±1.09	17.42±0.96	8.14±0.93	18.19±3.03	17.38±0.95	18.56±2.91	19.98±2.76	21.76±3.46	20.24±2.54	21.12±2.91
G-II	17.12±0.97	17.70±1.37	17.76±1.04	16.71±1.03	18.79±1.47	18.74±1.43	17.73±1.41	18.82±1.46	20.34±2.64	20.90±2.77	21.16±2.33	21.54±2.31
G-III	16.87±1.05	17.23±1.01	17.31±1.17	17.45±1.02	17.83±2.07	17.79±2.70	17.69±1.31	17.73±2.19	18.99±2.08	19.87±2.40	19.05±2.17	19.37±2.71

G-IV	17.3±0.90	17.7±1.01	17.9±1.27	17.1±0.92	18.5±1.39	18.9±2.29	17.4±1.09	18.6±2.20	20.1±2.37	21.2±3.03	20.6±2.32	21.1±2.53
G-V	17.8±0.67	18.4±0.89	18.8±1.78	18.2±0.66	18.3±1.07	20.9±1.98	19.1±0.69	20.4±2.23	20.4±2.01	21.2±3.45	21.3±2.22	21.4±2.18
F0 female rats												
G-I	16.8±1.22	17.0±3.45	17.3±5.98	17.4±4.12	18.1±1.76	18.3±9.16	18.1±8.68	18.4±7.00	18.8±8.22	19.5±8.71	19.1±4.11	19.3±2.34
G-II	16.7±6.68	17.1±2.34	17.4±5.04	17.5±1.33	18.0±9.32	18.5±4.23	18.4±9.41	18.7±1.07	19.1±6.15	19.9±2.68	19.6±7.36	19.5±3.09
G-III	15.0±3.12	16.0±3.04	16.1±6.14	17.2±5.12	17.8±3.11	17.1±9.22	17.9±9.42	17.0±9.15	17.7±6.17	17.5±4.36	17.0±7.23	17.1±2.44
G-IV	16.3±6.42	16.9±5.82	17.1±6.18	17.4±3.83	18.0±6.36	18.5±2.18	18.1±5.57	18.4±8.14	19.0±1.20	19.7±8.62	19.3±8.22	19.3±6.30
G-V	16.8±3.67	17.6±2.43	17.6±6.57	18.5±3.75	18.4±9.23	18.9±4.12	19.9±5.76	19.6±4.35	19.2±5.26	19.9±9.72	19.6±2.16	19.4±8.31

- Data are presented as mean±SDM, g/day/rat; One-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

Table 21: Food intake of F1 male and female parental animals during premating and mating periods following treatment with *M. stenopetala* leaf extracts

Groups	Premating										Mating	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
F1 male rats												
G-I	16.34±0.35	17.35±1.53	17.82±2.00	18.19±1.71	18.22±0.93	18.65±2.14	19.45±1.33	20.32±2.33	20.18±2.05	21.63±3.12	21.21±2.13	21.45±1.42
G-II	16.12±0.67	17.23±1.11	17.91±1.13	18.17±1.22	18.62±2.52	18.78±1.87	19.63±1.28	20.14±2.08	20.47±2.31	21.55±2.99	21.35±2.61	21.82±2.27
G-III	15.49±0.08	16.19±1.23	17.07±2.22	17.66±1.17	17.44±2.13	17.56±2.34	18.39±1.41	19.69±2.17	19.86±2.23	20.48±2.45	20.72±2.14	20.38±2.33
G-IV	16.18±0.41	17.44±1.27	17.93±2.17	18.88±1.43	18.42±2.49	18.75±2.11	19.64±1.34	20.33±2.22	20.56±2.19	21.60±2.84	21.15±2.28	21.61±2.14
G-V	17.15±0.54	18.00±1.21	18.01±2.31	19.98±1.63	18.80±1.41	19.19±2.07	20.08±1.34	20.71±2.31	21.21±2.17	22.23±2.78	22.11±2.23	22.77±2.55
F1 female rats												
G-I	15.67±0.48	16.38±1.02	18.41±2.12	17.99±1.71	17.87±2.42	18.32±2.51	19.16±1.41	19.71±1.97	19.42±2.08	20.33±2.17	20.67±2.22	20.34±2.09
G-II	15.89±0.55	16.27±1.37	18.08±2.11	17.87±1.22	17.72±2.33	18.13±2.22	19.28±1.30	19.56±2.15	19.33±2.16	20.44±2.04	20.43±2.17	20.25±2.12
G-III	14.64±0.34	15.73±1.22	16.91±2.19	16.66±1.17	16.63±2.61	17.90±2.93	18.14±1.26	18.32±2.21	17.91±1.19	19.99±2.03	19.11±2.23	19.97±2.31
G-IV	15.77±0.50	16.79±1.21	18.12±2.21	17.88±1.43	17.87±2.47	18.12±2.32	19.23±1.31	19.60±2.11	19.47±2.12	20.38±2.09	20.48±2.24	20.34±2.24
G-V	15.88±0.61	17.55±1.24	19.31±2.40	18.99±1.63	18.96±2.53	19.31±2.61	20.31±1.28	20.82±2.09	20.95±2.05	21.57±2.13	20.99±2.34	20.81±2.42

- Data are presented as mean±SDM, g/day/rat; One-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.2.2.2.Food intake During Gestation and Lactation Periods

Food intake was reduced in 1000 mg/kg treatment groups of F0 and F1 female rats during GD 0-7 and GD 7-14 than the pair-fed control groups. Furthermore, it was also reduced in the high dosage treated animals of both generations during lactation period. However, it was not statistically significant (Table 22).

Table 22: Food intake of F0 and F1 female parental rats during gestation and lactation periods following treatment with *M. stenopetala* leaf extracts

Food intake (g)	**Treatment groups**			**Control groups**	
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
F0 female rats					
No. of rats	20	18	17	20	20
Food consumption during gestation					
GD-0-7	19.42±3.40	18.20±1.02	17.15±0.69	18.87±1.92	20.72±2.57
GD-7-14	20.36±2.10	18.38±0.52	18.25±0.36	19.49±1.09	21.39±1.36
GD-14-21	21.40±1.59	20.26±1.23	21.51±2.55	21.17±1.55	21.50±0.81
Food consumption during lactation					
PND 1-7	27.23±2.24	28.89±4.22	24.23±1.03	27.83±2.41	30.95±2.13
PND 7-14	44.14±2.30	43.38±4.46	38.30±6.14	41.71±3.78	41.00±2.20
PND 14-21	45.55±2.43	42.99±4.06	44.69±3.38	44.53±3.12	44.88±2.59
F1 female rats					
No. of rats	20	20	19	20	20
Food consumption during gestation					
GD-0-7	19.24±2.03	18.52±2.19	17.29±1.08	18.82±1.68	20.38±1.43
GD-7-14	18.35±1.47	17.23±3.00	17.57±2.03	18.28±2.16	19.98±2.12
GD-14-21	21.18±2.06	20.97±2.61	20.89.34±2.35	21.10±2.36	21.37±2.42
Food consumption during lactation					
PND 1-7	24.48±2.61	24.76±3.51	22.57±3.09	24.50±2.89	26.18±2.33
PND 7-14	38.44±3.72	37.41±5.08	37.73±5.27	37.91±4.55	38.06±4.13
PND 14-21	43.29±6.53	41.52±5.07	41.34±4.66	42.43±5.12	43.56±4.32

- The findings are summarized as mean SDM; One-way ANOVA;
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.2.3. Body Weight of F0 and F1 Male and Female Parental Rats

5.2.3.1.Body Weight During Premating and Mating Periods

Throughout the premating and mating periods, the body weight of F0 and F1 parental animals of both sexes grew steadily in all groups. It was lower in 1000 mg/kg treated animals throughout the premating and mating periods, but these differences were not statistically significant (Figures 22 and 23).

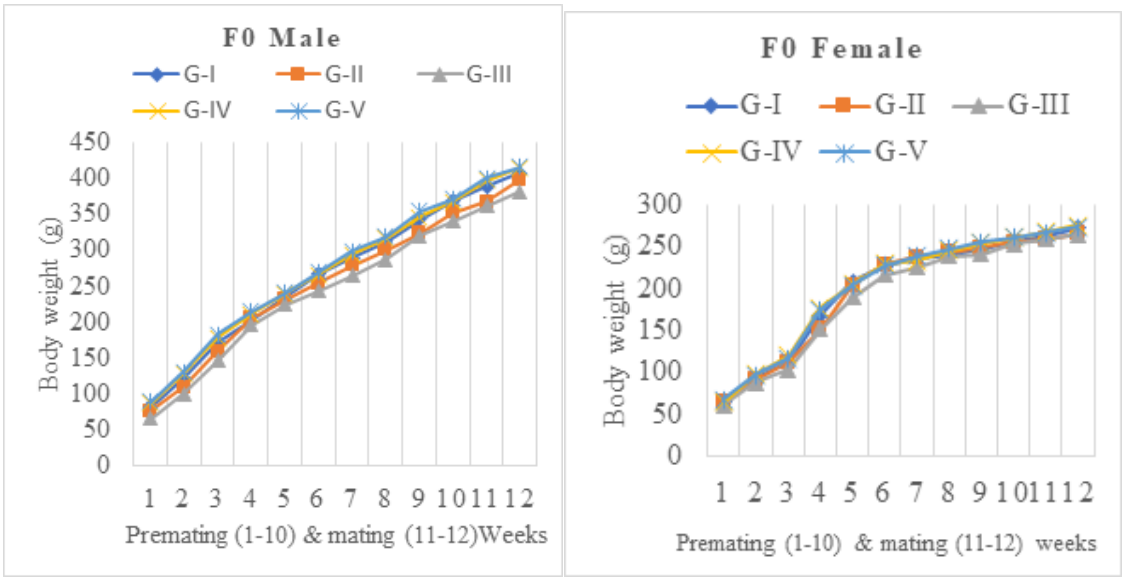


Figure 21: Body weight of F0 parental animals following treatment with *M. stenopetala* leaf extracts

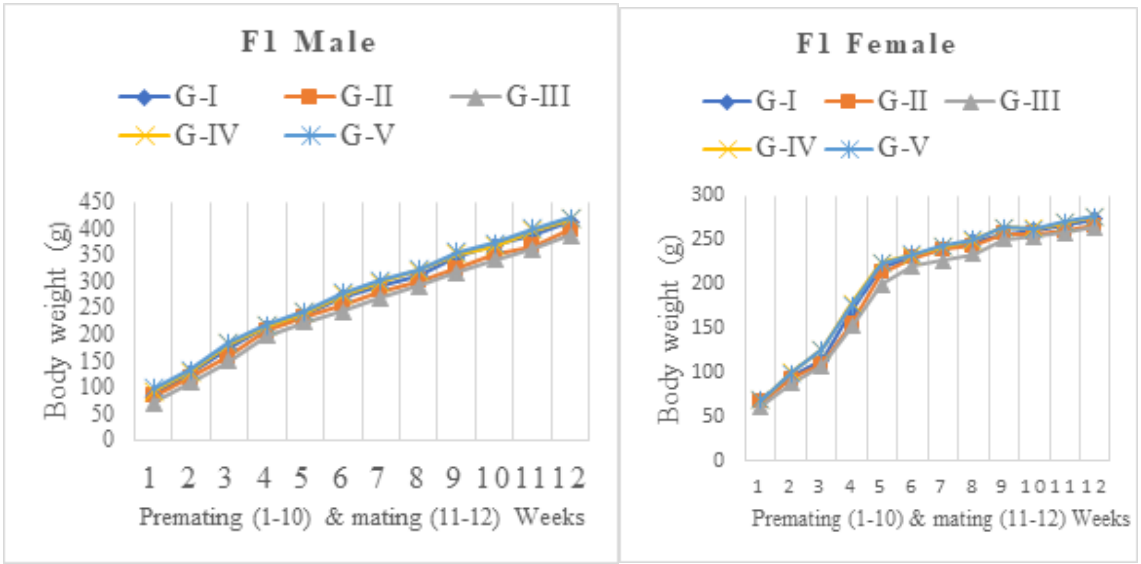


Figure 22: Body weight of F1 parental animals following treatment with *M. stenopetala* leaf extracts

5.2.3.2. Body Weight of F0 and F1 Female Rats During Gestation and Lactation Periods

Table 23 shows results of body weight measurement from F0 and F1 female rats during pregnancy and lactation periods. Maternal weight was significantly decreased in F0 and F1 female rats given 1000 mg/kg and 500 mg/kg of the test plant during gestation days 0, 7, 14, and 21. However, it was not statistically significant. Similarly, a significant difference in the maternal weight was not observed during the lactation period in both F0 and F1 female rats.

Table 23: Body weight measurement from F0 and F1 female rats during gestation and lactation periods following treatment with *M. stenopetala* leaf extracts

Body weight (g) on	**Parents**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of rats	F0	20	18	15	20	20
	F1	20	20	20	20	20
Gestation body weight						
GD-0	F0	270.15±14.39	260.25±3.58	259.25±4.98	276.33±26.71	280.00±6.65
	F1	274.36±9.09	264.15±11.42	262.25±12.12	280.35±4.28	282.53±6.56
GD-7	F0	287.07±27.03	276.25±3.93	276.05±3.95	292.06±13.30	294.75±5.95
	F1	286.75±7.55	279.12±9.26	278.54±10.15	296.52±7.75	298.21±6.76
GD-14	F0	300.44±11.62	291.25±2.94	291.30±3.04	307.25±4.94	308.80±17.41
	F1	299.25±6.62	296.23±11.62	295.12±8.43	310.09±9.25	312.12±8.16
GD-21	F0	349.89±10.82	345.30±6.19	343.25±5.68	354.25±4.88	356.47±16.57
	F1	352.32±7.65	348.47±10.8	346.23±11.0	357.43±11.3	358.10±10.2

			2	8	2	3
Lactation body weight						
PND 1	F0	299.25±8.06	298.67±8.76	297.33±13.09	300.00±8.74	299.25±10.92
	F1	304.55±9.34	302.75±6.09	300.22±9.11	303.55±6.52	307.33±7.06
PND 7	F0	281.75±10.19	283.28±10.34	279.40±16.53	280.25±12.40	281.25±14.47
	F1	290.55±6.23	287.37±8.56	286.12±11.67	289.13±9.17	290.36±10.16
PND 10	F0	291.75±6.46	293.83±18.56	290.33±13.44	289.50±9.19	290.70±11.17
	F1	293.06±7.12	289.47±12.33	289.26±9.56	292.18±10.21	293.10±12.24
PND 14	F0	300.75±4.94	295.33±15.22	296.53±14.87	304.00±8.11	300.70±11.49
	F1	301.15±6.38	298.45±12.19	294.63±9.06	300.09±7.32	303.12±9.61
PND 21	F0	302.50±4.26	295.11±14.33	301.47±12.76	303.00±8.43	302.50±10.14
	F1	304.32±8.38	298.99±11.06	299.35±8.37	304.12±10.13	306.36±12.03

- The findings are summarized as mean±SDM.
- GD: gestation day; PND: postnatal day; one-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.2.4. Reproductive Performance and Fertility of F0 and F1 Parental Rats

5.2.4.1. Evaluation of Estrous Cycle

In this study, the four phases and the length of the estrous cycle were evaluated using the types of cells observed in the vaginal smear test. In comparison to the pair-fed control groups, the estrous cycle was longer in 1000 mg/kg treated F0 and F1 female rats. In addition, the high dose and middle dose treated animals had a lower proportion of females with a normal estrous cycle. However, the differences did not reach statistical significance (Table 24).

Table 24: Length of estrous cycle and normality of F0 and F1 female rats following treatment with *M. stenopetala* leaf extracts

Parameters	**Treatment groups**			**Control groups**	
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
F0 parental rats					
No. of females examined	20	20	20	20	20
Females with normal estrous cycles (%)	90	85	85	100	100
Length of estrous cycles (days/dam)	4.7±0.66	4.8±0.70	5.2±0.62	4.4±0.50	4.3±0.47
F1 parental rats					
No. of females examined	20	20	20	20	20
Females with normal estrous cycles (%)	100	90	90	100	100
Length of estrous cycles (days/dam)	4.5±0.47	4.5±0.65	4.9±0.33	4.2±0.30	4.1±0.47

- Data are presented as mean ±SDM; One-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.2.4.2.Evaluation of Reproductive Indices

Findings from reproductive parameters evaluations are summarized in Table 25. A total of 100 female and 100 male rats were matched for each the F0 and F1 parental study (M: F; 1:1). Each group had a pair of twenty male and female rats.

All F0 both sexes of parental animals treated 250 mg/kg of the plant extract and all control groups displayed signs of successful mating and copulation. However, throughout the two-week mating period, one pair (5%) in the middle dose and two pairs (10%) in the high dose treated groups, did not show sign of copulation. As a result, the copulation index of the groups treated with 500 mg/kg and 1000 mg/kg was 95% and 90%, respectively.

In this study, all the mated F0 female parental animals were pregnant were pregnant, except one (5%) rat from the high dose treated animals. This showed that the fertility index of F0 female rats administered 1000 mg/kg of plant extract was 85%. In addition, one mated rat in the high dose treated group failed to conceive. Therefore, the fecundity index was lower in 1000 mg/kg treated animals (94.4%) than in the pair-fed control group (100%), however, it was not statistically significant.

The pre-coital time (the number of days stayed to be inseminated since pairing) was longer (3.5±0.95 days) in the high dose treated animals as compared to the pair-fed (2.4±0.94 days) control group, however, not statistically significant. In addition, no significant difference in the gestation length between the treatment and control groups.

However, a significant reduction in the gestation index was seen in 500 mg/kg and 1000 mg/kg administered animals and it was 94.7% and 88.2%, respectively when compared with the low dose treated and the paired-fed control groups.

In addition, an abortifacient effect was observed in 500 mg/kg and 1000 mg/kg treated animals. One pregnant rat from 500 mg/kg treated group underwent an abortion on the 17^{th} day of the gestation period. Additionally, two pregnant rats from 1000 mg/kg treatment group were aborted on the 16^{th} and 17^{th} days gestation period. In comparison with the low dose treated and the pair-fed control groups, a significantly increased (11.8%) abortifacient index was observed in 1000 mg/kg treated animals.

The mean number of implantations per dam and the total number of implantations in the high-dose treatment group were both statistically fewer than in the pair-fed control group. Post-implantation loss was also higher (10.8%) in the 1000 mg/kg treatment group

compared to the 250 mg/kg treated (5.3%) and pair-fed (6.4%) control groups, however, this difference was not statistically significant.

In F1 parent rats, all the paired male and female rats were successfully mated except one pair (5%) in the high-dose treated group. The pre-coital time and post-implantation loss were increased in the high-dose treatment group compared with the pair-fed control group. The abortifacient index was also increased (5.8%) in the high-dose treated group compared with all the treated and control groups, but not statistically significant. However, the mean number of implantations per dam and total number of implantations were significantly decreased in the 1000 mg/kg treated group compared with the 250 mg/kg treated, and pair-fed control groups. Otherwise, there were no significant differences in the copulation index, fertility index, fecundity index, gestation length, gestation index, or abortifacient index between the control and *M. stenopetala*-treated groups.

Table 25: Reproductive parameters of F0 and F1 parental animals treated with *M. stenopetala* leaf extracts

Reproductive indices	**Parents**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of paired; male/female	**F0**	20/20	20/20	20/20	20/20	20/20
	F1	20/20	20/20	20/20	20/20	20/20
No. of mated (paired)	**F0**	20	19	18	20	20
	F1	20	20	19	20	20
Copulation index (%) [a]	**F0**	100	95	90	100	100
	F1	100	100	95	100	100
Fertility index (%) [b]	**F0**	100	95	85	100	100
	F1	100	100	95	100	100
Fecundity index (%) [c]	**F0**	100	100	94.4	100	100
	F1	100	100	100	100	100
Pre-coital time (days) [e]	**F0**	2.9±0.79	3.2±1.24	3.5±0.95	2.4±0.94	2.2±0.70
	F1	2.9±0.43	3.0±0.74	3.4±0.65	2.7±0.36	2.6±0.76
Gestation length (days)[e]	**F0**	22.0±0.89	21.9±0.76	21.4±0.68	21.6±0.68	21.7±0.67
	F1	21.3±0.6	21.1±0.8	20.8±0.3	21.6±0.4	21.5±0.4

		4	1	7	9	4
Gestation index (%) [d]	**F0**	100	94.7	88.2**	100	100
	F1	100	100	94.7	100	100
Abortifacient index (%) [f]	**F0**	0	5.3	11.8**	0	0
	F1	0	0	5.8	0	0
No. of implantations/dam [e]	**F0**	9.5±2.23	9.5±2.48	8.2±2.04 *	10.9±1.7 4	10.6±1.4 3
	F1	10.5±3.1 6	9.3±2.63	8.3±2.17 *	10.7±2.0 4	10.8±1.7 3
Total no. implantation	**F0**	189	180	139*	220	211
	F1	210	186	158*	214	216
Total Post implantation loss (%) [g]	**F0**	5.3	6.7	10.8	6.4	1.4
	F1	3.2	3.2	4.3	3.3	1.9

- The findings are presented as the number, percentage, and mean ± SDM
- *significantly different from pair-fed control animals
- **significantly different from 250 mg/kg and pair-fed control animals (p value < 0.05); One-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

a. (No. of females mated/no. of females placed with males) ×100.
b. (No. of females pregnant/no. of females placed with males) ×100.
c. (No. of females pregnant/no. of females mated) ×100.
d. (No. of females with live pups/no. of females pregnant) ×100.
e. Values are means ± SDM; statistical test: Kruskal–Wallis + Mann–Whitney U test.
f. (No. of aborted rats/no. of pregnant rats) ×100.
g. Total post-implantation loss = ((No. of implantation sites−no. of pups born alive)/no. of implantation sites) ×100.

5.2.4.3.Effects on Pregnancy Outcomes

In this study, data regarding the pregnancy outcomes that collected from F0 and F1 parental animals are presented in Table 26. The number live births per dam as well as the total number of live births were significantly decreased in the high dose treated animals than the pair-fed control groups. The delivery index in the high dose treated animals was 89.2%. Otherwise, differences were not seen in the mean number of male and female pups, sex ratios or the number of postnatal deaths among the treated and control groups.

Table 26: Birth outcomes of F0 parental rats treated with *M. stenopetala* leaf extracts

Pregnancy outcomes	**Parental**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of rats	**F0**	20	18	15	20	20
	F1	20	20	19	20	20
No. of pups delivered alive/dam	**F0**	8.95±2.78	9.33±2.82	8.26±2.98 *	10.3±2.47	10.4±2.05
	F1	10.2±1.64	9.1±2.06	8.1±2.53*	10.5±2.61	10.6±3.15
No. of pups delivered alive/group	**F0**	179	168	124*	206	208
	F1	203	182	153*	210	211
No. of pups delivered dead/group	**F0**	7	8	5	4	3
	F1	4	3	4	3	2
No. of pups delivered dead/dam	**F0**	0.35±0.49	0.44±0.77	0.33±0.51	0.20±0.41	0.15±0.49
	F1	0.20±0.41	0.15±0.49	0.21±0.43	0.15±0.49	0.10±0.78
Delivery index (%) [a]	**F0**	94.7	93.3	89.2	93.6	98.6
	F1	96.7	97.9	96.8	98.1	97.7
No. of male/dam	**F0**	4.15±1.69	4.55±1.57	4.2±1.40	4.7±1.45	4.9±1.32
	F1	5.10±1.43	4.05±1.32	4.14±1.41	4.71±1.61	5.00±1.44
No. of female/dam	**F0**	5.15±1.09	5.22±1.25	4.4±1.58	5.9±1.02	5.7±0.73
	F1	5.25±1.16	5.20±1.27	5.22±1.36	5.94±1.13	5.65±1.06
Male: Female ratio/dam	**F0**	0.81:1	0.87:1	0.93:1	0.83:1	0.87:1
	F1	0.97:1	0.78:1	0.79:1	0.79:1	0.88:1

- The results are presented as number, mean ±SDM, percentage and ratio
- *Significantly different from the pair-fed control group; (p value < 0.05);
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- [a] Delivery index (%) = (No. of live pups delivered/no. of implantations) ×100.

5.2.5. Evaluation of Developmental Parameters of F1 and F2 Pups

5.2.5.1. Effects on Postnatal Survival of F1 and F2 Pups

The viability index of the F1 and F2 letters was evaluated on lactation days 0, 4 and 21. As shown in Figure 24, the viability index of the F1 and F2 litters on PND 4 was decreased significantly in 500 mg/kg and 1000 mg/kg treated animals as compared to the pair-fed animals. Otherwise, significant differences were not observed at PND 0 and PND 21 between the treatment and control groups of both generations.

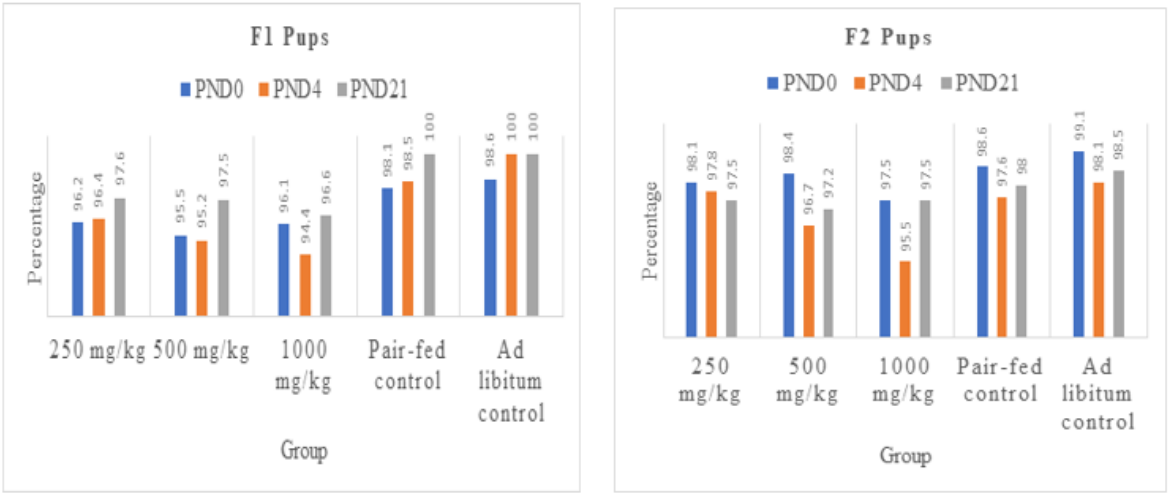


Figure 23: Viability index during lactation (%) of F1 and F2 letters following exposure with *M. stenopetala* leaf extracts; Viability index on PND0 (%) = (No. of live pups delivered/total no. of pups delivered) × 100; Viability index on PND4 (%) = (No. of live pups survived on day 4 of lactation/No. of live pups delivered) × 100; Viability index on PND 21 (%) = (No. of live pups survived on day 21 of lactation/No. of live pups survived on day 4 of lactation) × 100; PND: postnatal day.

5.2.5.2. Evaluation of Lactational Weight of F1 and F2 Pups

There were no significant variations in the pup's weight that was measured on PND 0, 4, 7, 14, and 21, between the treatment and control groups in either F1 or F2 pups (Table 27).

Table 27: Lactational weight of F1 and F2 pups following exposure of parent rats with *M. stenopetala* leaf extracts

Weight (g)	Sex	Pups	Treatment groups			Control groups	
			Group I	Group II	Group III	Group IV	Group V
PND 0	**M**	**F1**	6.39±0.20	6.48±0.21	6.39±0.36	6.67±0.45	6.94±0.55
		F2	6.23±0.41	6.33±0.42	6.42±0.55	6.72±0.45	6.89±0.61
	F	**F1**	6.37±0.22	6.18±0.36	6.14±0.24	6.27±0.32	6.37±0.30
		F2	6.03±0.34	6.06±0.71	6.12±0.33	6.15±0.02	6.05±0.40
PND 4	**M**	**F1**	9.06±0.39	9.08±0.33	9.00±0.86	8.75±0.85	9.09±0.35
		F2	9.16±0.12	9.23±0.43	9.20±0.37	9.06±0.45	9.19±0.26
	F	**F1**	8.68±0.97	8.37±1.02	8.34±0.92	8.34±0.84	8.54±0.80
		F2	8.78±0.77	8.67±0.12	8.71±1.04	8.66±0.63	8.79±0.23
PND 7	**M**	**F1**	15.11±1.10	15.26±1.78	14.53±1.53	14.66±1.59	15.24±1.28
		F2	14.93±2.00	14.97±1.65	15.03±0.91	14.99±2.07	15.06±1.67
	F	**F1**	12.96±2.65	11.90±1.84	11.98±0.88	12.05±1.73	12.38±2.18
		F2	11.94±3.00	12.01±1.43	12.03±0.31	12.11±2.03	11.99±1.97
PND 14	**M**	**F1**	29.82±1.23	28.66±3.32	27.81±2.67	27.67±2.64	28.09±3.25
		F2	28.95±1.46	28.90±2.95	28.55±1.26	28.54±2.12	28.39±2.98
	F	**F1**	26.56±2.94	23.18±4.65	24.28±4.25	24.40±3.87	24.58±4.44
		F2	24.24±1.67	24.10±3.34	24.50±2.13	24.32±2.33	24.06±3.16
PND 21	**M**	**F1**	53.70±5.34	53.47±5.30	53.31±5.56	53.94±5.34	55.40±4.98
		F2	54.70±6.23	55.06±4.75	55.41±622	54.87±5.30	54.40±5.62
	F	**F1**	49.38±5.05	49.93±4.28	48.20±3.92	47.76±3.70	48.10±2.83
		F2	47.34±4.11	48.67±3.65	49.01±5.42	48.04±6.31	49.03±6.08

- The values are presented as mean ±SDM
- PND: postnatal day; M: male; F: female; one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

5.2.5.3.Effects on the Anogenital Distance of F1 and F2 Pups

The AGD of F1 and F2 pups was carefully measured on PND 0 and 4. In both F1 and F2 pups, the AGD was not vary significantly. The longest AGD was recognized in the *ad libitum* control groups at PND 0 and PND 4 in the F1 and F2 pups (Table 28).

Table 28: Anogenital distance of F1 and F2 pups following exposure with *M. stenopetala* leaf extracts

Anogenital Distance (mm)	**Sex**	**Pups**	**Treatment groups**			**Control groups**	
			Group I	**Group II**	**Group III**	**Group IV**	**Group V**
PND 0	**M**	**F1**	3.60±0.50	3.67±0.49	3.53±0.52	3.55±0.51	3.75±0.44
		F2	3.58±0.46	3.48±0.66	3.64±0.91	3.78±0.43	3.90±0.57
	F	**F1**	1.85±0.37	2.00±0.49	2.00±0.38	1.95±0.39	2.21±0.39
		F2	2.01±0.21	1.97±0.41	2.00±0.23	2.01±0.26	2.11±0.56
PND 4	**M**	**F1**	7.00±0.46	7.06±0.42	6.87±0.52	6.90±0.45	7.14±0.64
		F2	7.12±0.46	7.00±0.38	7.02±0.43	7.11±0.78	7.23±0.51
	F	**F1**	3.60±0.50	3.56±0.51	3.47±0.52	3.55±0.51	3.95±0.22
		F2	3.76±0.33	3.56±0.72	3.67±0.24	3.69±0.45	3.84±0.42

- The results are expressed as the mean ± standard deviation of the mean**,** one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- mm: millimeter; PND: postnatal day; M: male; F: female

5.2.5.4.Effects on the Postnatal Developmental Indices of F1 and F2 Pups

In the present study, the postnatal developmental statuses of F1 and F2 pups are presented in Table 29 and in Figures 25, 26 and 27. There were no significant differences in the age at which male and female F1 and F2 pups showed pinnal detachment, incisor eruption, hair sprouting, ear opening and eye opening between the control and *M. stenopetala* leaves extract-treated groups.

Table 29: Postnatal developmental indices (days) of F1 and F2 pups following exposure with *M. stenopetala* leaf extracts

Developmental index	**Sex**	**Pups**	**Treatment groups**			**Control groups**	
			Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of rats examined	**M/F**	**F1**	40/40	36/36	30/30	40/40	40/40
		F2	40/40	40/40	40/40	40/40	40/40
Days of pinnal detachment	**M**	**F1**	3.00±0.51	3.17±0.38	3.13±0.46	3.00±0.39	2.75±0.44
		F2	2.99±0.43	3.06±0.42	3.21±0.52	3.03±0.29	2.88±0.72
	F	**F1**	2.70±0.47	2.67±0.49	2.73±0.46	2.60±0.50	2.60±0.50
		F2	2.56±0.18	2.73±0.38	2.66±0.15	2.71±0.12	2.82±0.45
Days of incisor eruption	**M**	**F1**	11.10±0.31	11.11±0.47	11.00±0.38	11.05±0.39	10.85±0.37
		F2	11.05±0.35	11.15±0.56	11.11±0.24	11.11±0.41	10.67±0.64
	F	**F1**	11.15±0.37	11.06±0.24	10.93±0.26	10.95±0.22	10.95±0.39
		F2	11.07±0.41	11.12±0.33	11.12±0.31	11.05±0.35	10.77±0.46
Days of hair sprouting	**M**	**F1**	10.05±0.22	10.06±0.24	10.20±0.41	10.05±0.22	10.00±0.00
		F2	10.12±0.46	10.15±0.35	10.26±0.65	10.12±0.46	10.05±0.22

	F	**F1**	10.05±0.22	10.00±0.00	10.00±0.00	10.10±0.31	10.05±0.22
		F2	10.34±0.43	10.16±0.44	10.14±0.27	10.16±0.44	10.12±0.35
Days of ear opening	**M**	**F1**	17.00±0.46	17.06±0.24	17.00±0.00	16.95±0.39	16.90±0.45
		F2	16.87±0.34	16.96±0.27	17.06±0.44	17.01±0.32	16.89±0.67
	F	**F1**	17.20±.41	17.11±0.47	17.07±0.46	16.90±0.45	16.90±0.31
		F2	17.01±.36	17.07±0.65	17.11±0.34	17.05±0.56	16.96±0.27
Days of eye opening	**M**	**F1**	14.80±0.62	15.00±0.49	15.13±0.35	15.05±0.39	14.85±0.37
		F2	14.65±0.71	14.95±0.77	15.05±0.62	15.08±0.78	14.99±0.04
	F	**F1**	15.00±0.32	15.11±0.32	15.20±0.41	14.95±0.51	14.80±0.52
		F2	14.98±0.54	15.08±0.56	15.07±0.36	15.02±0.29	14.93±0.52

- The results are expressed as the mean ± standard deviation of the mean, one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- M: Male; F: Female;

Figure 24: Developmental stages of F1 pups from F0 female rats treated with the high dose of *M. stenopetala* leaf extracts; 1: on PND 0, 2: on PND 4, 3: on PND 7, 4: on PND 14, and 5: on PND 21.

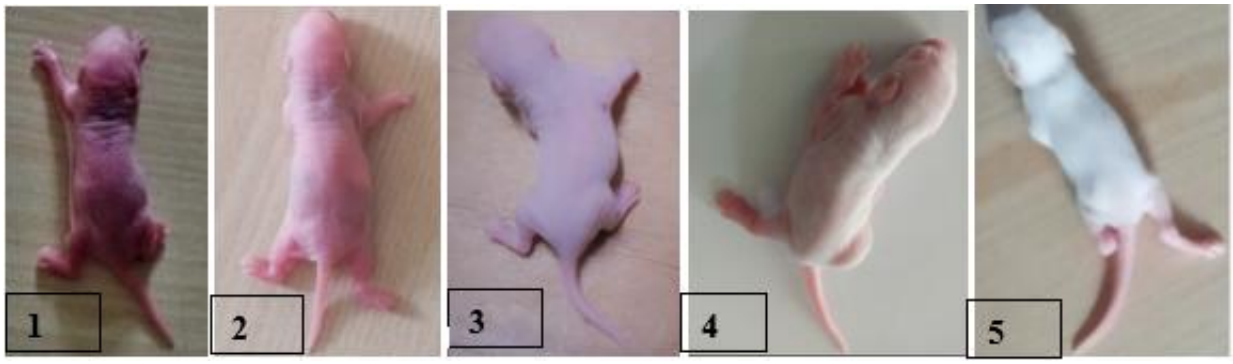


Figure 25: Developmental stages of F2 pups from F1 female rats treated with the high dose of *M. stenopetala* leaf extracts; 1: on PND 0, 2: on PND 4, 3: on PND 7, 4: on PND 14, and 5: on PND 21.

Figure 26: Developmental stages of F1 pups from the pair-fed control group of F0 female rats; 1: on PND 0, 2: on PND 4, 3: on PND 7, 4: on PND 14, and 5: on PND 21.

5.2.5.5.Effects on Sexual Maturation of F1 and F2 Pups

In the current study, data regarding sexual maturation of F1 and F2 pups are summarized in Table 30. Significant differences were not seen in the age at preputial separation in males, vaginal opening in females, or the mean body weight at the age of preputial separation or vaginal opening in F1 and F2 pups between the treatment and control groups.

The age of the preputial separation in F1 male rats ranged between 35 and 56 days, and the mean days of preputial separation in the high-dose treated and pair-fed control groups were 42.87±2.47 and 41.80±1.67 days, respectively. In addition, the age of vaginal opening in the F1 female rats ranged between 26 and 38 days.

In F2 male pups, the age of preputial separation was also ranged between 37 and 56 days. The mean days of preputial separation in the high-dose treated and pair-fed control groups were 42.76±2.23 and 40.65±2.44 days, respectively. The age of vaginal opening in the F2 female rats also ranged between 26 and 38 days.

Table 30: Sexual maturation of F1 and F2 pups following exposure of with *M. stenopetala* leaf extracts

Parameters	**Pups**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of Rats; M/F	**F1**	40/40	36/36	30/30	40/40	40/40
	F2	40/40	40/40	40/40	40/40	40/40
Days of preputial separation	**F1**	43.15±3.17	42.72±3.74	42.87±2.47	41.80±1.67	42.40±2.23
	F2	42.05±2.02	42.13±2.63	42.76±2.23	40.65±2.44	41.45±2.42
Weight at preputial separation (g)	**F1**	166.15±12.42	169.28±10.04	172.60±6.62	172.25±6.42	169.85±11.11
	F2	172.23±11.33	170.34±12.23	171.41±7.43	170.35±9.36	171.67±10.52
Days of vaginal opening	**F1**	32.10±3.13	31.56±4.06	32.53±2.36	31.25±2.22	30.45±2.52
	F2	33.23±2.05	32.41±3.15	32.44±3.16	32.18±3.04	31.33±1.06
Weight at vaginal opening (g)	**F1**	122.10±5.59	123.56±7.59	126.20±7.44	125.35±6.58	123.50±5.73
	F2	126.11±7.39	124.66±8.49	125.35±8.65	125.74±8.68	126.34±6.67

- The findings are stated as mean±SDM, One-way ANOVA
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control; M: Male; F: Female.

5.2.6. Serum Level of Reproductive Hormones in F0 and F1 Parental Rats

In the high dose treated animals of both F0 and F1 male parent rats, testosterone, FSH, and LH levels were significantly higher than in the pair-fed control group. Furthermore, when compared to the pair-fed control group, testosterone levels were significantly higher in the 500 mg/kg treated group of both F0 and F1 male parent rats. Moreover, F0 and F1 female parent rats had higher serum levels of LH, FSH, progesterone, and estradiol in the high-dose treated group than in the pair-fed control group, but these differences were not statistically significant (Table 31).

Table 31: Serum hormonal levels in F0 and F1 male and female parental rats treated with *M. stenopetala* leaf extracts

Parameters	**Parents**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
Male parental rats						
No. of Animals	**F0**	20	20	20	20	20
	F1	20	20	20	20	20
LH(MIU/ml)	**F0**	0.291±0.01	0.297±0.01	0.308±0.01*	0.293±0.01	0.289±0.01
	F1	0.292±0.03	0.296±0.01	0.310±0.01*	0.291±0.01	0.292±0.02
FSH (ng/ml)	**F0**	5.58±2.46	5.53±2.44	6.52±2.71*	5.55±2.42	5.56±2.62
	F1	5.60±3.00	5.60±2.06	6.59±2.81*	5.58±2.13	5.58±2.47
Testosterone (pg/ml)	**F0**	4.97±0.98	5.54±1.40*	6.06±1.26*	4.51±1.37	4.23±1.30
	F1	5.54±0.86	6.24±1.17*	7.11±0.72*	5.40±1.28	5.36±1.35
Female parental rats						
No. of Animals	**F0**	20	20	20	20	20
	F1	20	20	20	20	20
LH (MIU/ml)	**F0**	0.319±0.01	0.341±0.01	0.396±0.03	0.299±0.01	0.306±0.02
	F1	0.315±0.01	0.345±0.00	0.365±0.01	0.306±0.00	0.307±0.01
FSH (ng/ml)	**F0**	4.23±1.75	4.39±2.03	4.58±2.11	4.20±2.08	4.09±2.33
	F1	4.72±2.13	4.70±2.45	5.73±2.66	4.71±2.31	4.74±2.37
Progesterone (ng/ml)	**F0**	31.44±5.79	33.22±16.18	33.99±16.27	31.13±19.80	30.96±11.37

	F1	28.69±11.25	31.20±12.19	32.99±10.46	29.46±10.46	31.55±13.43
Estradiol (pg/ml)	**F0**	21.22±5.46	20.78±13.73	22.71±6.36	21.67±13.19	19.09±3.19
	F1	28.60±22.51	31.06±22.79	32.97±19.61	30.90±22.79	28.47±22.30

- The results are expressed as the mean ± standard deviation of the mean.
- *Significantly different from the pair-fed control group; one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- FSH, follicle-stimulating hormone; LH, luteinizing hormone.

5.2.7. Sperm Count, Motility and Morphology in F0 and F1 Parent Rats

The sperm parameters for F0 and F1 male rats are shown in Table 32. When compared to the low dosage treated and the pair-fed control groups, the number of sperm cells and percentage of sperm motility were slightly increased in the high dose treated group of F0 and F1 male parent rats. A decreased percentage of abnormal sperm cells was also seen in the high-dose treated groups of both F0 and F1 parent rats. However, they were not statistically significant. The number of sperm cell counts and motility in the F1 parent rats were somewhat higher in all *M. stenopetala* leaves extract treated groups compared to the F0 parent rats. Furthermore, the proportion of abnormal sperm cells was reduced in all the treatment groups of F1 parent rats compared with the F0 parent rats treated the plant extract. Nevertheless, it was not statistically significant.

5.2.8. Gross Investigation and Weight of Reproductive Organs from F0 and F1 Parent Rats

In the current investigation, the reproductive organs of F0 and F1 male and female parent rats were thoroughly examined for any grossly visible anomalies before being microscopically examined. No gross abnormalities were seen in any of the reproductive organs of the treatment or control groups of either F0 or F1 parent rats. The male and female reproductive organs analysed in this investigation are shown in Figure 28.

Table 32: Results of sperm analysis in F0 and F1 male parent rats treated with *M. stenopetala* leaf extracts

Groups	No. of animals	Parameters					
		F0 parent rats			F1 parent rats		
		SC (10^6/ml)	SM (%)	AM (%)	SC (10^6/ml)	SM (%)	AM (%)
G I	**20**	189.35±18.60	79.19±12.32	10.35±2.68	191.15+12.79	81.23±11.09	8.55±3.02
G II	**20**	187.80±16.53	80.78±9.33	9.95±3.80	189.55+26.17	82.43±10.67	9.35±2.60
G III	**20**	193.85±15.30	83.43±6.35	8.15±4.22	197.05+13.61	85.08±12.17	6.15±2.00
G IV	**20**	188.25±17.61	77.09±11.04	10.05±4.30	191.35+16.60	79.43±8.51	8.60±2.33
G V	**20**	189.25±18.11	78.52±7.66	9.70±4.13	192.30+24.19	80.07±13.07	8.85±2.48

- Results are stated as the mean ± standard deviation of the mean, one-way ANOVA.
- Abnormal sperm morphology (double head, absent head, mis-shaped heads, absent tail, curved tail).
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- SC; sperm count, SM; sperm motility, AM; abnormal morphology

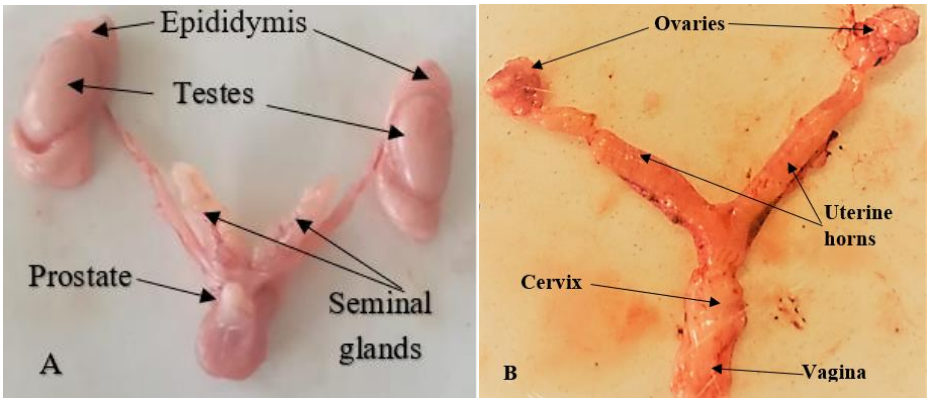


Figure 27: Male (A) and female (B) reproductive organs of rats given the higher dose of *M. stenopetala* leaf extracts

On the day of sacrifice, the body weights and reproductive organ weights of F0 and F1 male and female rats were measured. In addition, the relative organ weight was calculated by dividing the absolute organ weight by the rat weight at necropsy and multiplied by a hundred. There were no significant differences in the absolute and relative organ weights of the testes, epididymis, prostate gland, and seminal glands between the treatment and control groups of F0 and F1 parent rats. Furthermore, neither the absolute nor relative weights of the uterus nor the ovary differed significantly between the treatment and control groups of both F0 and F1 female rats (Table 33).

Table 33: Absolute and relative organ weight of F0 and F1 male and female parent rats treated with *M. stenopetala* leaf extracts

Weight	**Sex/Organs**	**Parents**	**Treatment groups**			**Control groups**	
			Group I	**Group II**	**Group III**	**Group IV**	**Group V**
Final body weight (g)	**Male**	**F0**	365.10±28.28	363.11±8.14	400.13±17.22	395.40±15.61	399.50±15.82
		F1	401.60+31.15	389.40+16.85	392.50+14.63	396.30+21.96	391.50+11.68
	Female	**F0**	292.00±11.89	294.60±23.42	298.10±22.69	293.60±15.30	293.60±17.38
		F1	272.90+24.99	260.50+15.85	274.50+11.80	268.70+17.38	273.30+10.33
Absolute organ weight (g)	**Testes**	**F0**	1.46±0.16	1.47±0.07	1.48±0.14	1.46±0.14	1.46±0.12
		F1	1.54+0.10	1.55+0.07	1.56+0.04	1.55+0.15	1.43+0.08
	Epididymis	**F0**	1.15±0.11	1.15±0.05	1.16±0.08	1.16±0.07	1.17±0.06
		F1	1.25+0.14	1.14+0.10	1.12+0.13	1.21+0.25	1.38+0.20
	Prostate	**F0**	0.77±0.09	0.71±0.11	0.63±0.11	0.65±0.09	0.64±0.09

	gland	**F1**	0.72+0.10	0.69+0.10	0.68+0.11	0.69+0.09	0.71+0.13
	Seminal glands	**F0**	0.60±0.09	0.58±0.13	0.52±0.07	0.52±0.06	0.53±0.06
		F1	0.56+0.13	0.53+0.10	0.49+0.04	0.50+0.07	0.53+0.13
	Ovaries	**F0**	0.15±0.02	0.15±0.02	0.15±0.01	0.15±0.01	0.17±0.03
		F1	0.16+0.03	0.15+0.02	0.15+0.03	0.16+0.02	0.17+0.04
	Uterus	**F0**	0.64±0.14	0.69±0.23	0.68±0.10	0.72±0.15	0.76±0.05
		F1	0.62+0.11	0.54+0.20	0.52+0.10	0.60+0.09	0.59+0.14
Relative organ weight (g)	**Testes**	**F0**	0.38±0.05	0.37±0.02	0.37±0.03	0.37±0.03	0.37±0.02
		F1	0.39+0.03	0.40+0.02	0.40+0.02	0.39+0.03	0.37+0.02
	Epididymis	**F0**	0.34±0.05	0.31±0.01	0.29±0.02	0.29±0.02	0.29±0.02
		F1	0.31+0.04	0.29+0.03	0.28+0.03	0.30+0.06	0.35+0.05
	Prostate	**F0**	0.21±0.03	0.20±0.03	0.16±0.03	0.16±0.02	0.16±0.02
		F1	0.18+0.03	0.18+0.03	0.18+0.03	0.17+0.03	0.18+0.04
	Seminal	**F0**	0.17±0.02	0.16±0.04	0.15±0.02	0.16±0.01	0.16±0.01
		F1	0.14+0.03	0.14+0.02	0.12+0.01	0.25+0.03	0.14+0.04
	Ovaries	**F0**	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.06±0.00
		F1	0.06+0.01	0.06+0.01	0.06+0.00	0.06+0.01	0.06+0.02
	Uterus	**F0**	0.22±0.01	0.23±0.01	0.22±0.01	0.23±0.00	0.24±0.00
		F1	0.23+0.05	0.21+0.08	0.22+0.04	0.22+0.04	0.22+0.05

- The results are stated as the mean ± standard deviation of the mean, one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control
- F0: F0 parents; F1: F1 parents; g: gram.

5.2.9. Histopathological Findings in the Reproductive Organs of F0 and F1 Parental Rats

In the current study, histopathological findings were examined for F0 and F1 parent rats. A light microscopic examination of Hematoxylin and eosin-stained tissues of the testis, epididymis, prostate gland, and seminal glands in males, uterus, ovary and vagina in females were performed.

5.2.9.1. Findings from Male Reproductive Organ Histopathology (F0 and F1 Parental Rats)

A histological examination of the testes from F0 and F1 parent rats did not reveal any spermatogenic cells or spermatid loss, nor did it reveal any seminiferous tubule degeneration. The testicular histopathology in the treatment (Figure 29a, b) and control (Figure 29c, d) groups did not significantly differ from one another.

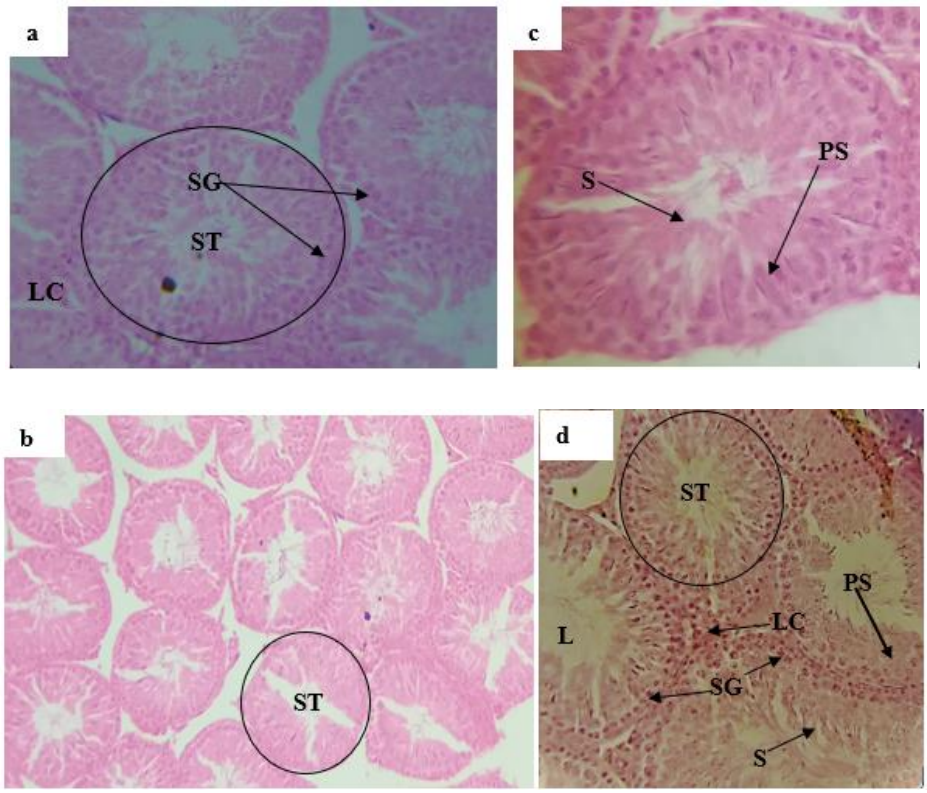


Figure 28: Photographs of testis showing normal microscopic structures. Sections were taken from F0 (a) and F1 (b) males rats given 1000 mg/kg *M. stenopetala* leaf extracts and control groups (c & d). ST: Seminiferous tubule, SG: Spermatogonium, PS: Primary spermatocyte, S: Spermatid and LC: Leydig cells; E and H stain, a & b using 40x, and c & d using 100x magnification.

Similarly, the photomicrographs of the epididymis in the treatment (Figure 30a, b) and control (Figure 30c, d) groups of F0 and F1 parent rats did not show any apparent differences. In each of the treatment and control groups, it was possible to see the thick epithelial lining on the wall and sperm cells within the tissues' lumen.

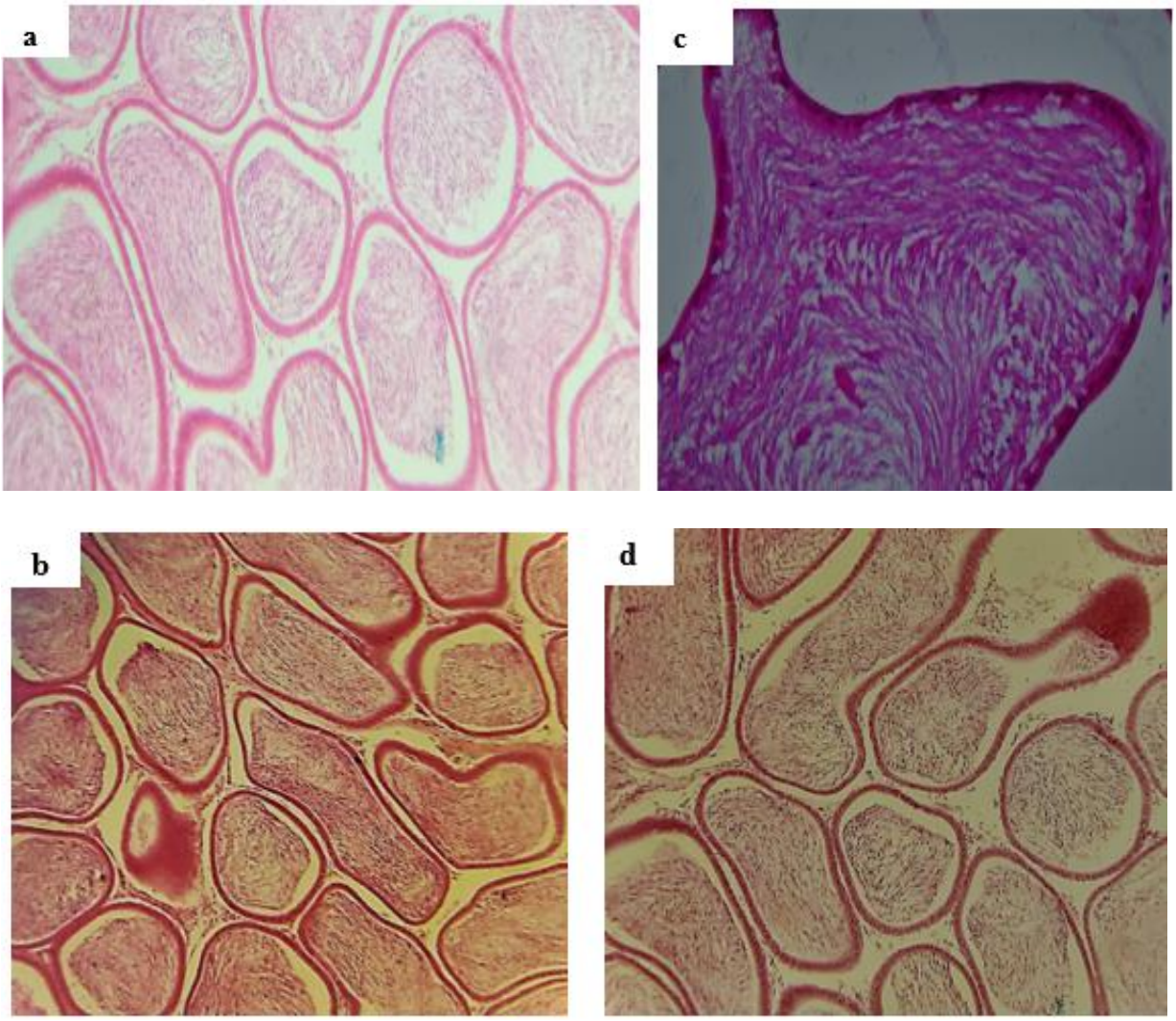


Figure 29: Photomicrograph of the epididymis showing normal microscopic structures. Sections were taken from F0 (a) and F1 (b) male rats given 1000 mg/kg *M. stenopetala* leaf extracts and control groups (c and d). E and H stain, a, b, d using 40x and c using 100 x magnification.

Furthermore, the stroma, lumen, and epithelium of the prostate gland of both F0 and F1 parent rats were microscopically evaluated. Between the treatment and control groups, there were no alterations in the fibromuscular stroma, duct system, or secretory part. In each of the treatment (Figure 31a, b) and control (Figure 31c, d) groups, normal epithelial lining and glandular secretion-filled lumens were observed.

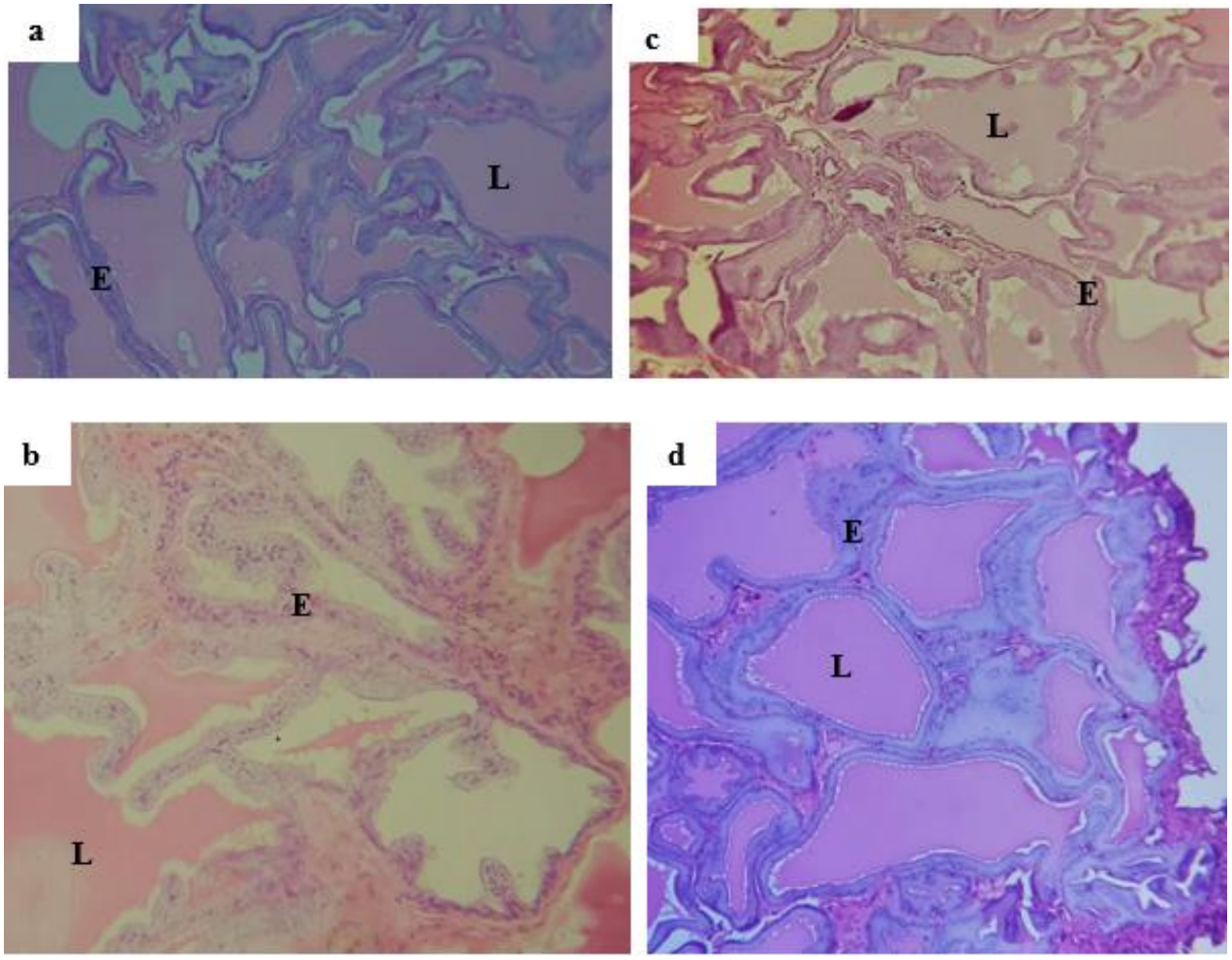


Figure 30: Photographs of the prostate gland showing normal secretory epithelium and stroma. Sections were taken from F0 (a) and F1 (b) male rats given 1000 mg/kg *M. stenopetala* leaf extracts and control groups (c & d). L: Lumen, E: Epithelium; E and H stain, using 40x magnification.

Similarly, there were no discernible alterations between the treatment (Figure 32a, b) and control (Figure 32c, d) groups in the photomicrographs of the seminal glands. The epithelium, lumen, and fibromuscular tissues did not exhibit any abnormalities.

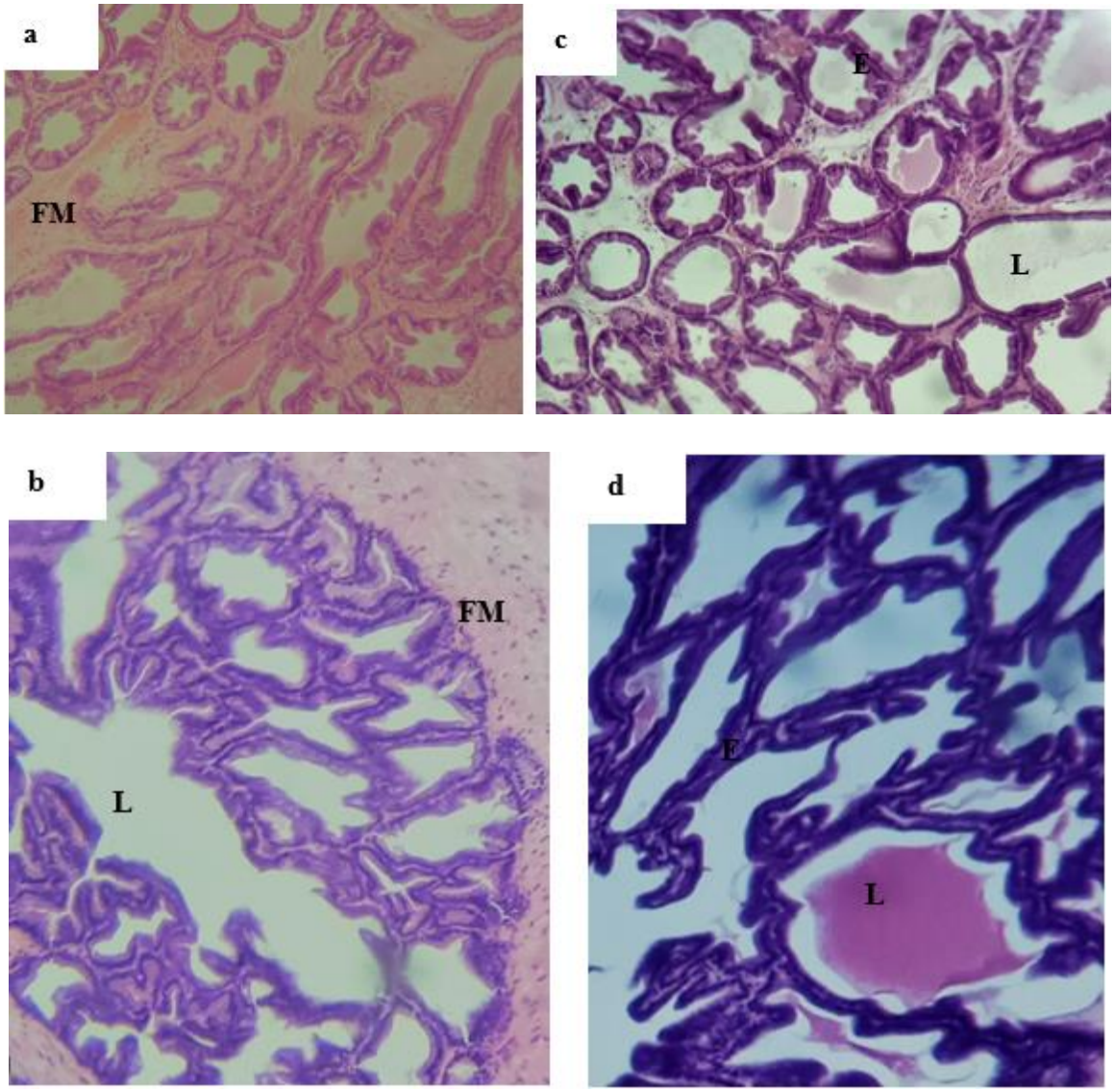


Figure 31: Photograph of the seminal gland showing normal secretory epithelium and stroma. Sections were taken from F0 (a) and F1 (b) male rats given 1000 mg/kg *M. stenopetala* leaf extracts and control groups (c & d); FM: Fibromuscular, L: Lumen, E and H stain, using 100x magnification.

5.2.9.2. Findings from Histopathology of Female Reproductive Organs (F0 and F1 Parent Rats)

In the current investigation, the ovaries, uterus, and vagina were also examined using light microscopy. According to the observations, there were no appreciable differences in the ovarian, uterine, or vaginal tissues between the treatment and control groups of F0 and F1 females. Nevertheless, one F0 female rat treated with 1000 mg/kg of the test plant showed hypertrophied vaginal epithelium (Figure 35a).

Figure 33 displays the morphometric analyses of the ovaries. In both the F0 and F1 females administered the high dosage of the test plant (a & b) and the pair-fed control (c & d) groups, normal ovarian tissues were seen. The corpus luteum, ovarian follicles at various stages of development, cuboidal germinal epithelium as well as the stroma of the medulla and its contents were seen.

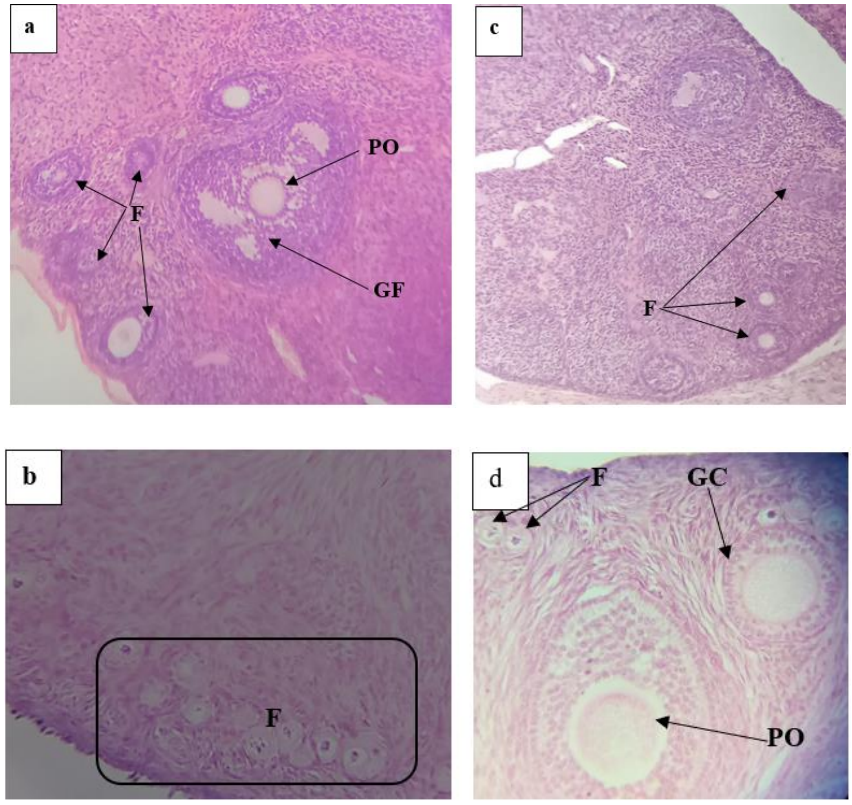


Figure 32: Photograph of ovarian tissues that show normal ovarian tissues that were taken from F0 (a) and F1(b) rats administered the high dose of *M. stenopetala* leaf extracts and pair-fed control groups (c & d). F: Follicles, GC: Granulosa cells, GF: Graafian follicle and SO: Secondary oocyte; E and H stain, a, b, c using 40x and d using 100x magnification.

Furthermore, there were no significant differences between the treatment and control groups of F0 and F1 parent rats in the endometrial glands and uterine epithelial lining. In the uterine myometrium and perimetrium of the experimental (Figure 34a, b) and control (Figure 34c, d) groups, no structural alterations were observed.

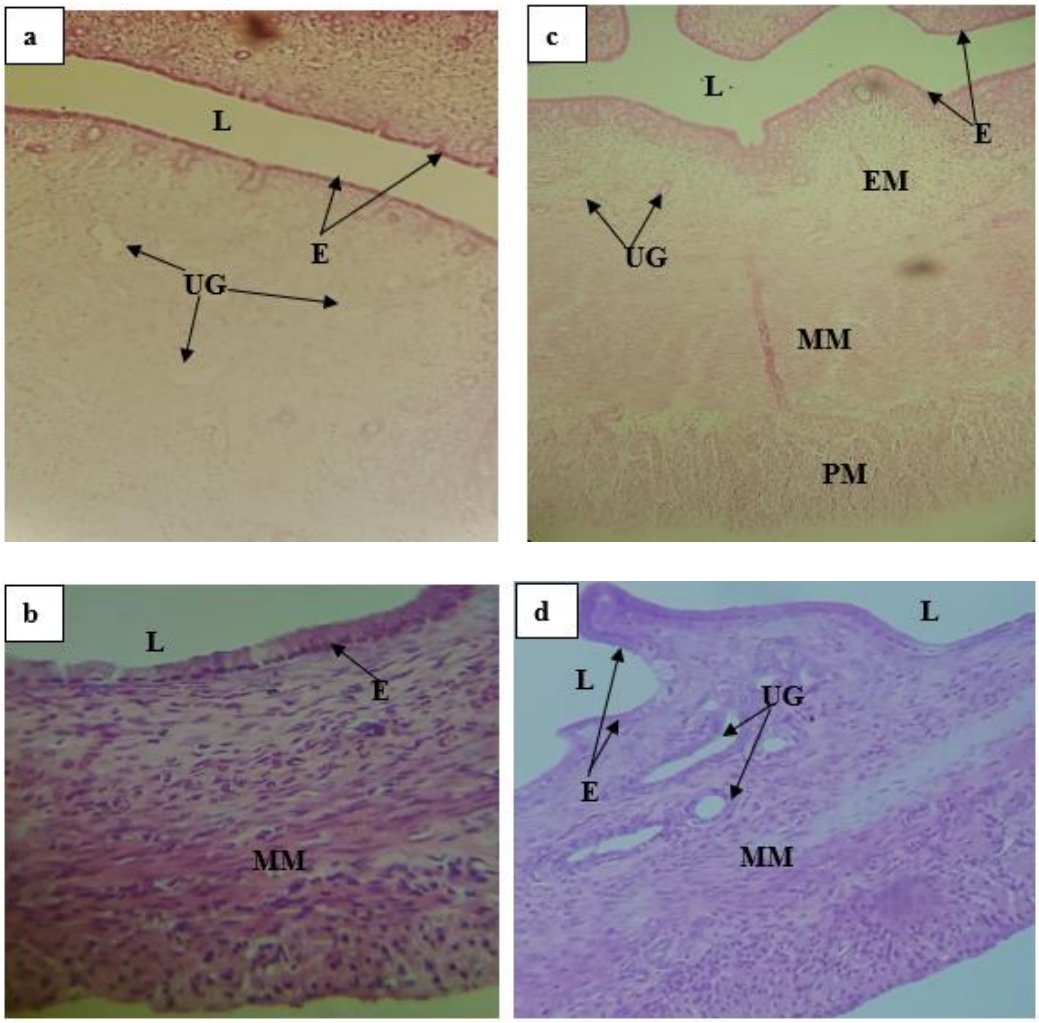


Figure 33: Photograph of uterine tissues that show normal uterine epithelium and musculature that were taken from F0 (a) and F1 (b) rats given the high dose of *M. stenopetala* leaf extracts (a) and pair-fed control groups (c & d). L: Uterine lumen, E: Epithelium, UG: Uterine glands, EM: Endometrium, MM: Myometrium and PM: Perimetrium; E and H stain, a & c using 40x, b & d using 100x magnification.

Additionally, the vaginal epithelium, musculature, and adventitia did not reveal a significant difference between the treatment and pair-fed control groups of F0 and F1 female rats (Figure 35 b, c & d).

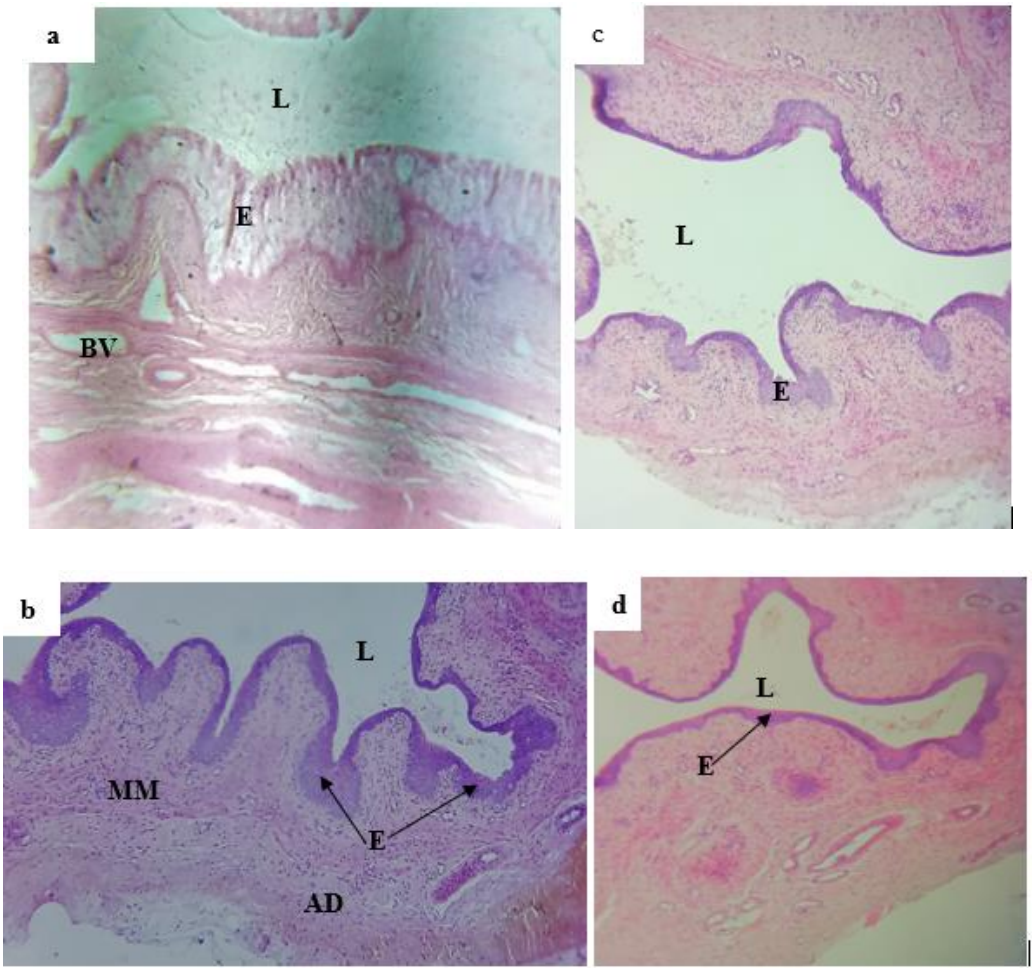


Figure 34: Photograph of vaginal tissues that show hypertrophied vaginal epithelium that was reserved from F0 rat administered the high dose of *M. stenopetala* leaf extracts (a), revealed normal vaginal epithelium, musculature, and adventitia that was taken from F1 rats treated the high dose of the test plant extract (b) and from F0 (c) and F1 (d) pair-fed control groups. L: Vaginal lumen, E: Epithelium, AD: Adventitia and MM: Muscle; E and H stain using 100 x magnifications.

5.2.10. Serum Biochemical Profiles of F0 and F1 Parental Rats

In the current study, tables 34 and 35 provide the findings of serum clinical chemistry tests from F0 and F1 parental rats. In F0 male parental rats, a statistically significant rise in the serum ALT and creatinine levels was seen in the 1000 mg/kg treatment group when compared with the low- and middle-dose treated and pair-fed control groups. In addition, compared to the low dosage treated (0.28±0.51) and pair-fed (0.29±0.03) control groups, the level of creatinine was significantly increased in the 500 mg/kg (0.33±0.41) treated group. However, the level of urea was significantly lowered in the high-dose treated group

than the low- and middle-doses treated and pair-fed control groups. Furthermore, compared to the pair-fed control group, the glucose levels in all the treatment groups were significantly reduced. Apart from the above changes, there were no noticeable differences between the treatment and control groups in any other serum parameters (Table 34).

In the high-dose treated F1 male parental rats, there were a significantly increased HDL levels compared to the pair-fed control group. However, a significant decreased in the level's LDL was seen in the high dose treated groups than in the pair-fed control animals. In addition, compared to all the treatment and control groups, the glucose level in the high-dose-treated F1 parental rats was also significantly decreased. In comparison to the pair-fed control groups, the glucose level was also significantly reduced in the 250 mg/kg- and 500 mg/kg-treated F1 male parental rats. However, no other blood parameters of the treatment and control groups of F1 male parental rats were significantly different from one another (Table 34).

Table 34: Serum biochemical profile of F0 and F1 male rats given *M. stenopetala* leaf extracts

Parameters	**Parents**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of Animals	**F0**	20	20	20	20	20
	F1	20	20	20	20	20
ALT (IU/L)	**F0**	78.95±15.23	78.51±12.50	87.26±8.37*	77.47±10.64	76.41±13.40
	F1	56.80±5.75	53.80±0.00	49.075±10.99	60.64±13.91	55.89±3.49
AST (IU/L)	**F0**	172.04±26.00	173.88±24.50	173.48±28.11	171.13±18.26	172.82±18.51
	F1	107.70±46.37	207.30±0.00	209.58±77.56	207.46±69.52	105.97±64.01
ALP (IU/L)	**F0**	88.00±24.07	90.00±3.08	83.00±9.51	89.00±0.00	94.60±20.70
	F1	107.50±2.56	106.00±0.00	103.25±19.98	103.05±15.18	111.65±5.58

GGT	**F0**	3.80±1.77	5.35±2.46	4.10±1.40	5.00±4.10	4.20±3.40
(IU/L)	**F1**	4.32±1.05	4.44±1.63	4.42±1.32	4.37±2.00	4.33±2.13
Urea	**F0**	40.83±6.63	39.75±0.36	33.40±1.72 *	38.95±2.72	38.56±4.35
(mg/dl)	**F1**	41.80±2.77	42.30±0.00	41.23±1.89	42.35±1.48	41.81±2.49
Creatinine	**F0**	0.28±0.51	0.33±0.41**	0.38±0.03*	0.29±0.03	0.28±0.04
(mg/dl)	**F1**	52.90±3.39	52.00±0.00	51.50±3.45	53.38±6.72	52.59±5.07
Total	**F0**	62.98±7.15	59.40±1.44	57.53±3.44	56.80±7.79	62.20±9.09
cholesterol	**F1**	54.23±3.63	52.33±2.43	51.63±3.23	56.42±4.06	55.68±7.12
(mg/dl)						
Total	**F0**	5.99±0.48	6.01±0.66	6.03±0.73	5.98±0.45	5.88±0.69
protein	**F1**	6.52±1.34	6.49±1.08	6.58±1.56	6.43±1.27	6.59±1.42
(g/dl)						
Glucose	**F0**	97.40±58.89 *!	86.63±39.13*!	79.45±10.21*!	116.58±47.35	113.45±49.13
(mg/dl)	**F1**	140.90±35.50*!	128.10±0.00*!	114.57±33.27*	172.40±57.21	185.01±47.97
Albumin	**F0**	4.62±0.36	4.43±0.10	4.46±0.11	4.42±0.22	4.50±0.30
(g/dl)	**F1**	3.92±0.07	3.95±0.00	3.98±0.13	4.12±0.12	4.05±0.25
HDL	**F0**	27.70±4.62	33.70±0.31	31.18±3.00	29.40±5.54	28.72±5.26
(mg/dl)	**F1**	33.30±3.08	32.30±0.00	38.88±1.17 *	32.80±4.34	31.43±3.36
LDL	**F0**	21.56±3.43	24.77±2.33	23.86±1.97	23.45±4.02	22.98±2.04
(mg/dl)	**F1**	20.43±2.98	22.33±2.41	17.52±3.21 *	21.63±2.51	22.77±2.31
Sodium	**F0**	145.00±1.03	143.50±2.50	144.15±1.69	144.60±2.56	145.30±1.03
(mEq/L)	**F1**	147.50±0.51	147.00±0.00	146.75±1.68	146.40±0.94	145.90±2.22
Chloride	**F0**	104.00±0.92	103.70±3.79	103.38±2.78	102.95±2.31	103.37±1.95
(mEq/L)						

	F1	105.75±0.46	107.00±0.0 0	107.43±1.5 0	106.10±1.5 3	104.68±0.7 9
Potassiu m (mEq/L)	**F0**	5.26±0.27	5.31±0.63	5.09±0.64	5.21±0.01	5.23±0.17
	F1	6.26±0.87	5.55±0.00	5.16±0.41	5.59±0.10	6.11±0.7
Calcium (mEq/L)	**F0**	2.50±0.31	2.49±0.12	2.46±0.13	2.49±0.12	2.49±0.22
	F1	2.14±0.04	2.07±0.00	2.13±0.05	2.15±0.07	2.16±0.13
Phosphat e (mEq/L)	**F0**	3.07±1.22	2.67±1.15	2.98±1.29	2.43±0.84	2.95±1.29
	F1	2.68±0.31	2.74±0.00	2.76±0.25	2.81±0.29	2.80±0.33
Iron (mEq/L)	**F0**	2.24±0.08	2.28±0.09	2.21±0.16	2.13±0.18	2.11±0.18
	F1	2.20±0.05	1.99±0.00	2.09±0.19	2.11±0.06	2.12±0.07

- The results are expressed as the mean ± standard deviation of the mean,
- *Significantly different from all treated and control groups (Tukey test)
- **Significantly different from the 250 mg/kg treated and pair-fed control groups (Dunnett test)
- $^{*!}$ Significantly different from the pair-fed control group.
- For all p values < 0.05; one-way ANOVA,
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

In the current investigation, F0 female rats were found to have significantly different levels of the liver injury indicators like ALT, AST, and ALP. When compared with all other treatment and control groups, ALT (140.33±46.86) and ALP (186.25+20.70) levels were significantly increased in the 1000 mg/kg treated group. In addition, the AST levels were significantly increased in the middle dose treated and high dose treated animals. Likewise, serum ALP levels were significantly increased in the 500 mg/kg treated group (162.00±28.18) compared with the low-dose treated and pair-fed control groups. Furthermore, the creatinine level was significantly elevated, whereas the urea level was significantly decreased in F0 females treated with 500 mg/kg and 1000 mg/kg of the test plant.

Compared with the pair-fed control group, the glucose level was significantly reduced in F0 females treated with 500 mg/kg and 1000 mg/kg of the plant extract. Similarly, when compared with the low dose treated and pair-fed control groups, the HDL level was elevated significantly in the high- and middle-dose treated groups. Nevertheless, compared to all other groups, the LDL levels were significantly decreased in the 1000 mg/kg treated rats. Unless otherwise noted, significant differences were not observed in the other serum biochemical parameters between the treatment and control groups of F0 female rats (Table 35).

Similar to F0 female rats, significant differences in the levels of liver injury biomarkers were observed between the treatment and control groups of F1 female parent rats. The ALT levels were significantly elevated in the 500 mg/kg (79.35±26.26) and 1000 mg/kg (110.53±16.33) treated rats. In addition, the AST levels were significantly increased in the middle (224.10±1.03) and high (239.59±53.16) dose treated rats. Moreover, the ALP levels were significantly increased in the 500 mg/kg (177.90±72.71) and 1000 mg/kg (198.50±3.60) treated groups. Furthermore, the level of urea was significantly increased in the middle and higher dose treated group than the low-dose treated, and pair-fed control groups. However, the glucose level was significantly decreased in the high-dose treated group than the low-dose treated and pair-fed control groups, (Table 35).

Table 35: Serum biochemical profile of F0 and F1 female rats given *M. stenopetala* leaf extracts

Parameters	**Parents**	**Treatment groups**			**Control groups**	
		Group I	**Group II**	**Group III**	**Group IV**	**Group V**
No. of Animals	**F0**	20	20	20	20	20
	F1	20	20	20	20	20
ALT (IU/L)	**F0**	112.38±10.94	122.95±24.67	140.33±46.86*	120.48±15 2.32	118.03±18.15
	F1	71.75±8.80	79.35±26.26**	110.53±16.33*	68.35±28.06	65.20±12.43
AST (IU/L)	**F0**	185.34±31.25	219.78±70.82**	217.39±12.88**	180.22±26.04	177.81±25.38
	F1	204.3±39.12	224.10±1.03**	239.59±53.16*	199.41±56.90	198.91±48.52

ALP (IU/L)	**F0**	145.85±63.13	162.00±28.18**	186.25±20.70*	144.60±41.53	152.75±109.15
	F1	135.40±62.99	177.90±72.71**	198.50±3.60**	133.93±79.4	125.00±15.39
GGT (IU/L)	**F0**	34.55±3.43	32.75±4.99	65.25±25.82	85.60±28.36	39.00±21.45
	F1	1.50±3.59	-1.5±0.51	0.80±2.14	0.20±1.51	-1.25±1.21
Urea (mg/dl)	**F0**	50.80±2.41	46.46±6.31	44.52±4.46***	51.68±9.02	56.35±0.99
	F1	47.35±9.94	46.09±13.07	45.80±0.00	43.92±6.53	44.65±3.23
Creatinine (mg/dl)	**F0**	131.18±31.27***	141.60±14.76***	146.60±60.92***	111.28±19.71	113.30±54.27
	F1	77.10±3.59	80.90±2.57	79.88±21.59	76.18±17.02	79.64±11.99
Total cholesterol (mg/dl)	**F0**	59.51±2.11	60.31±2.09	59.87±4.17	59.66±4.23	58.98±4.34
	F1	58.09±4.19	57.26±3.28	58.12±3.57	59.03±4.18	58.98±4.21
Total protein (g/dl)	**F0**	7.04±2.22	6.99±1.23	6.87±2.04	6.92±2.31	6.96±1.71
	F1	6.52±2.61	6.43±2.16	6.44±2.03	6.51±2.34	6.47±1.42
Glucose (mg/dl)	**F0**	125.90±40.30	113.76±7.87**	109.76±9.73**	128.43±26.41	126.98±17.48
	F1	102.20±57.15	98.30±2.26	92.53+19.54***	107.82±23.25	100.73±14.49
Albumin (g/dl)	**F0**	4.46±0.52	4.48±0.45	4.48±0.31	4.91±0.23	4.61±0.67
	F1	4.25±0.21	4.72±0.09	4.37±0.86	4.51±0.57	4.18±0.25
HDL (mg/dl)	**F0**	102.58±24.48	124.85±20.74**	126.90±44.78**	113.14±13.04	110.58±41.15
	F1	54.12±27.58	56.245±18.37	58.95±5.08	64.85±0.05	64.96±15.14
LDL (mg/dl)	**F0**	31.21±4.73	32.01±3.25	24.42±4.66*	32.27±4.33	31.99±4.61
	F1	29.72±3.26	30.03±4.63	31.31±3.91	29.95±4.11	30.12±4.35

Sodium (mEq/L)	**F0**	144.00±2.58	145.50±1.15	146.00±1.62	146.05±4.02	144.75±1.52
	F1	149.00±1.03	157.00±1.03	152.53±8.37	152.40±5.60	153.65±2.41
Chloride (mEq/L)	**F0**	104.27±2.45	105.05±0.86	104.60±0.65	104.90±1.80	105.65±1.35
	F1	106.25±2.00	104.45±1.59	107.39±2.70	106.45±1.89	105.19±0.83
Potassium (mEq/L)	**F0**	4.66±0.25	5.93±1.43	4.84±0.32	5.02±0.37	4.73±0.23
	F1	6.73±0.53	8.05±0.07	8.37±1.42	7.60±0.97	7.17±0.14
Calcium (mEq/L)	**F0**	2.53±0.08	2.28±0.37	2.16±0.18	2.30±0.13	2.14±0.11
	F1	2.07±0.08	2.12±0.08	2.12±0.22	2.14±0.18	2.10±0.08
Phosphate (mEq/L)	**F0**	2.00±0.11	2.05±0.69	1.79±0.30	1.77±0.14	1.92±0.20
	F1	3.09±0.04	3.46±0.03	3.12±0.21	3.17±0.12	2.54±0.51
Iron (mEq/L)	**F0**	2.24±0.10	2.16±0.40	2.10±0.27	2.33±0.19	2.18±0.51
	F1	3.47±0.27	3.47±0.42	3.62±0.65	4.04±0.44	4.02±0.15

- The results are expressed as the mean ± standard deviation of the mean,
- *Significantly different from all treatment and control groups,
- **Significantly different from 250 mg/kg treated and pair-fed control groups
- ***Significant difference from the pair-fed control group
- All p values < 0.05; one-way ANOVA.
- group I: 250 mg/kg, group II: 500 mg/kg, group III: 1000 mg/kg, group IV: pair-fed control, group V: *ad libitum* control

CHAPTER SIX

6. DISCUSSION

Several parts of medicinal plants, including their stems, leaves, flowers, roots, fruits , and seeds, are often employed to manage different types of diseases [146]. The extensive usage of herbal medicine is currently rising in both industrialized and developing nations, and it is attracting much attention worldwide [12]. Due to modern treatments' inability to effectively treat several chronic conditions, as well as their accessibility and affordability, medicinal herbs have become increasingly popular. Despite their obvious advantages, many of the chemical components found in herbal remedies are naturally poisonous and teratogenic to both humans and animals [147]. Additionally, research on the toxicity profiles and teratogenicity of the raw and various phytochemical components of therapeutic plants is minimal or non-existent [148].

The African Moringa, also known as *M. stenopetala*, is recognized for its therapeutic and nutritional values [11]. On various plant sections, many researches have been conducted to identify its acute, sub-chronic as well as chronic toxicities. However, studies on its teratogenic potential as well as developmental and reproductive toxicity are limited and the findings are inconsistent. Consequently, the current work examined the potential teratogenicity of ethanol extract of *M. stenopetala* leaf on 12 days old rat embryos and 20 days old rat fetuses. In addition, the study examined the plant's reproductive toxicity in Wistar albino rats over two generations.

6.1.The teratogenic potential of *M. stenopetala* leaf

In this study, in-vivo embryotoxicity and fetotoxicity of *M. stenopetala* leaf extract was evaluated. To conduct this teratogenicity study, the plant material was given to the pregnant rats during the pregnancy days 6 through 12, where organogenesis and embryogenesis is actively takes place in rodents. The morphological scores were used to evaluated the teratogenicity of the plant on the developmental status of the body systems on 12 days old rat embryos. In addition, the histology of the placenta, visceral organ development, and skeletal development of the fetuses were all examined to determine whether the plant extract caused teratogenic effects on the near-term rat fetuses at gestation day 20.

In this study, there was no deaths or noticeable behavioural changes between the treatment and control groups throughout the course of the experiment. In the day 12 experiment, the body weight and daily food consumption was decreased in the treatment groups. In addition, the body weight was decreased in the high dose treated animals as compared to the pair-fed control animals. Yet, this was not statistically significant. Besides to this, in the day 20 experiment, the daily feeding and body weight in the high dose treated pregnant rats were decreased during the treatment and posttreatment periods, but not statistically significant. This result is in agreement with an earlier study report that stated insignificant variations in the maternal daily food consumption or body weight between the mice treated various doses of *M. stenopetala* leaves extract and control group of mice [149]. However, results of our investigation were inconsistent with other previous studies reported a significant reduction of maternal bogy weight and food intake in pregnant rats treated different doses of *M. stenopetala* leaves [60, 150]. The justification for this decrease in the maternal food consumption and changes in the maternal body weight were remain constant, and it appears likely to blame toxicological features [151]. In addition, the other justification for changes in the body weight and food consumption of the animals could be toxicity, progression of disease, or a reaction of the animals to the treatment [27, 152]. Furthermore, the other claim for these alterations might be due to the harmful effect exerted on the epithelial lining of the digestive tract by tannins one of the constituents of *M. stenopetala* leaf, and caused reduction of absorption of nutrients and further reduce food intake that leads to reduction of weight gain [27]. A reduction in the body weight of animals treated extracts from the same plant species is reported in another previous stud [100].

In this study, maternal daily food consumption and weight gain during the treatment and posttreatment periods were decreased in the pair-fed control groups when compared to the ad libitum control group. The probable justification for these decreases might be due to manipulation of the animals in the pair-fed control group during the administration of distilled water that induce stress that leads to decrease the maternal daily food intake and weight gain.

In addition, food restriction in the pair-fed control group might be the other reason for this result. The rats in the pair-fed control group were restricted from fed and they provided food that was restricted and matched to the amount of food consumed by the experimental animals. But, animals in the *ad libitum* group were untouched and were not treated and not

manipulated throughout the experiment and fed freely. So, touching and manipulation of animals during the experiment might bring stress and disturb the behaviour of the animals and affect food consumption and maternal weight gain. The findings of the current study agreed with the reports of previous study that stated an incidence of stress induced reduction of body weight in rats [149].

Seyoum *et al.* [121] state that the somite number, the CRL, the yolk diameter, and morphological scores of the developing rat embryos are used as a significant parameters to identify and determine the stage of embryonic growth. Furthermore, the developmental stage and age of the embryos are directly correlated with morphological scores [124].

In this investigation, all animals treated the plant extract revealed a reduction in the number of somites, yolk sac diameter, CRL, and morphological scores when compared with the ad libitum and pair-fed control groups. When comparing pregnant rats treated 1000 mg/kg and 500 mg/kg to the pair-fed control group, all of the aforementioned indicators of embryonic development showed significant reductions. This finding aligns with another study that demonstrated a significant reduction in the morphological scores and somite number in the rat embryos subjected to *M. stenopetala* leaf extract treatment [100]. This could be due to the existence of alkaloids in the leaves of the plant that have the potential to be teratogenic for developing rat embryos and fetuses [91].

In the present study, the pregnancy outcomes of the pregnant rats treated the plant extracts showed variations. A significantly increased embryonic and fetal resorptions were observed in the high dose treated rats. In addition, the number of embryos and fetuses were decreased in the high dose treated animals. These results may indicate that the plant extract has disruptive or antiplantation effects on the zygote that has been implanted. Furthermore, the result is consistent with another study that found that pregnant rats given 80% methanol extract of *M. stenopetala* leaf experienced a high number of fetal resorptions [150]. Furthermore, it aligns with another study that designed to investigate effects of *M. oleifera* on the pregnant rat and discovered that it resulted in abortions [153]. These similar results could be explained by the similarities between plants in the Moringaceae family and their possible shared phytochemical components.

This is also analogues to the findings of another study designed to investigate embryonic and fetal developmental toxicity of *S. guineense* leaves by Abebe et.al. [128]. The probable justification for these comparable results might be because of the existence of alkaloids in

the leaves of *M. stenopetala* and *S. guineense* that might have a teratogenic effect on the developing rat embryos and fetuses [91]. In addition, reduction of the CRL, yolk sac diameter, somite number, and morphological scores suggested that the plant extract causes developmental retardation in the growing rat fetuses and embryos because these parameters have a linear relationship with the age of embryos and fetuses [124].

Delays in the development of the otic system (9.7%), somite scores (10.7%), and yolk sac circulation (4.9%) were identified in 1000 mg/kg treated animals as compared to the pair-fed control animals. Furthermore, the developmental status of the otic system and somite score were markedly delayed in 500 mg/kg treated animals. This result is in line with another previous studies that reported developmental delays of the yolk sac circulations, the sensory otic system [128], and somite scores in high-dose treated rat embryos [99, 100, 150].

However, the primordial heart, allantois, neurological system, optic system, olfactory system, craniofacial area, and musculoskeletal systems of the embryos did not exhibit developmental differences between the treatment and the control animals. Likewise, the developmental status of the brachial bars, maxillary process, mandibular process, and flexion of the embryos did not show significant difference between the treatment and control animals. Nevertheless, the current finding is inconsistent with a previous study that reported developmental delays in the branchial bars, mandibular process, optic system, olfactory system, and degree of flexion of the embryos of pregnant rats treated with 1000 mg/kg of the plant extract as compared to the pair-fed control group [150]. These discrepancies may be because of the differences of the parts of the plant studied or might be because of the differences in the solvents that were used to extract active molecules from the plant. In this study, 70% ethanol was used to extract the leaves of the plant, whereas 80% methanol was used to extract the seeds of the plant in the previous studies [99, 100, 150].

In the current study, indications for potential developmental retardation were observed in the near-term rat fetuses. A significant decrease in the CRL of the rat fetuses was observed in the high dose treated group. Furthermore, weights of the placenta and fetuses were significantly decreased in 1000 mg/kg treated animas than the pair-fed control animals. The rate of fetal resorption was also significantly increased in the higher dose treated pregnant animals. This developmental delays in the fetal growth might be due to the

alkaloids present in the *M. stenopetala* leaves that have potential to interfere with cholinergic neurotransmission, which results in developmental abnormalities in fetuses [90].

In the late pregnancy period, mineralization of the osseous tissue is one aspect of the development and an indication of fetal maturity [154]. In this consideration, ossification status of the near-term rat fetuses was investigated in the current study, following exposure of the pregnant animals to the test plant. In all the treated and control groups, the were no skeletal malformations in the skull bones, thoracic vertebrae, ribs hyoid bone. Yet, there were variations in the ossification centres in the metatarsus, metacarpus, forelimb phalanges, hind limb phalanges, sternebrae and sacro-cuadal vertebrae between the tread and control groups. However, these was not statistically significant. From the result, ethanol extract of M. stenopetala leaf might not exert that much adverse effect on the skeletal ossification during fetal development in rats.

The placenta is a temporary feto-maternal organ that forms a barrier between maternal and fetal circulation. It is an essential organ that facilitate the local exchange of important and also harmful materials between the maternal and fetal circulation that transport essential nutrients, gases, waste materials, and immunoglobulins. It also facilitates the exchange of chemical information, such as toxins and drugs, between the fetuses and the exposed mother. [101]. Because of this, it is the organ most vulnerable to direct toxic insults caused by chemicals; numerous toxic agents that affect the placenta have been reported. Thus, histopathological examination of the placenta is important for comprehending the mechanisms underlying developmental and embryotoxic effects, and it may also be advantageous for research on reproductive toxicity [155, 156].

In the current investigation, a significantly decreased placental weight was observed in 1000 mg/kg treated animals. Moreover, the treatment groups showed histopathological alterations such as capillary dilatation, decidual cellular apoptosis, hematoma filled the trophoblastic and labyrinthine zones, and decidual degeneration. But these changes were not statistically significant, except the trophoblastic proliferation. This is in line with other earlier research that looked into the toxic effects of medicinal plants and reported decreases in placental weight and histological changes associated with treatment [27, 100, 155]. The most likely cause of the decrease in placental weight and histological alterations could be the bioactive substances present in the plant under investigation, such as terpenoids, which

have the ability to cross the placenta and disrupt fetal development [90]. Another possibility might be the presence of terpenoids as well as alkaloids that might be related with increased osteogenic protein-1 levels, or increased bone morphogenetic protein-7 (BMP7), which is a type of transforming growth factor (TGF), in the placenta's decidua, which is located near the implantation site [157].

6.2.Two-generation Reproductive Toxicity

This two-generation reproductive toxicity research was designed to investigate the effects of the plant extracts on the integrity and performance of F0 and F1 male and female (F0 and F1 parents) reproductive organs as well as developmental milestones (F1 and F2 pups). It was intended to assess endpoints at certain periods, such unusual behaviour, reproductive outcomes, estrous cyclicity, and histopathological findings of reproductive organs. In addition, food consumption and body weight of the animals were assessed. Furthermore, the substantial physical developmental landmarks such as AGD, and parameters that help to determine sexual maturation status of F1 and F2 pups were evaluated. Sperm parameters like sperm count, sperm motility and morphology, serum biochemical and levels of reproductive hormones, were also evaluated in this study.

In this two-generation reproductive toxicity study, all of the F0 and F1 parental animals of both sexes survived the entire experimental duration. In either generation, there were no deaths during the whole experiment. Additionally, both generations showed consistent clinical findings. The treatment groups of the two generations did not show plant-related clinical symptoms of potential harm and behavioural abnormalities.

In this study, food consumption of F0 and F1 parental animals was reduced in the high dose treated animals during the premating ad mating periods. Similarly, it was reduced in 1000 mg/kg treated female animals during gestation ad lactation periods. However, it was not statistically significant. This reduced food consumption was going with the reduction of body weight of the treated animals. The reason for the reduction of food intake and weight the rats were consistent and it could be toxicologically significant [151]. The other justification for reduction of the body weight of the animals might be indication for the presence of toxicity or progression disease or might be response of the animals to the treatment [27, 152]. Beside to this, one of the secondary metabolites, tannins found in the leaves of *M. stenopetala* may damage the epithelium of the digestive tract and may reduce absorption of nutrients and further reduced food intake and then reduced weight of the

animals [27]. In another study, a reduction of body weight in animals treated plant extracts from the same plant species was reported [100].

Changes in the morphology of the reproductive organs, changes in the critical phases or length of estrous cycle, spermatogenesis, were all features of reproductive toxicity. These alterations could be regulate and determine the reproductive performance, hostile effects on the pregnancy outcomes, ad postnatal survival of the offspring [158]. This experimental study investigated the reproductive toxicity of *M. stenopetala* leaves extract in F0 and F1 rats, taking into consideration the estrous cycle length and the extent to which the pregnancy become successful. Furthermore, serum biochemical parameters, hormone levels, sperm count and morphology as well as histopathological examination of reproductive organs were also assessed.

The estrous cycle which is homologues with menstrual cycle in mammals is characterized by variations in the morphology of female reproductive organs such as the ovary, uterus, and vagina. The estrous cycle has four different phases named proestrus, estrous. metestrus and diestrus [159]. From the literature, the mean length of the estrous cycle is 4.5 days [160]. In the current study, the estrous cycles were longer in animals treated 1000 mg/kg body weight of the test plant than the animals in the pair-fed control groups both F0 and F1 parental animals. In the high dose and middle dose treated groups of F0 and F1 parental animals, increased percentage of estrous cycle abnormality was observed. However, it was not statistically significant. This tendency for the longer estrous cycles and abnormality may be evidence that the extract from *M. stenopetala* leaves affects rats' estrous cycles. This result is consistent with a study by Abebe *et al.* that reported that rats treated given a higher dosage level of *S. guineense* a plant with comparable constituents to the plant under investigation in this study, had an abnormal and a lengthy effect on the estrous cycle [27]. Even though the way the plant extract affects the estrous cycle is unknown, the disruptive effects caused by the secondary metabolites like flavonoids, tannins, and alkaloids in the crude extract of the tested plant may have an antigonadotropin effect and may reduce ovulation [27, 161].

In this study, the copulation, fertility, and fecundity indices were decreased in 1000 mg/kg treated animals than the pair-fed control groups. These were not statistically significant variations. This could be a sign that the plant extract has little potential for harming rat copulation, fertility, or fecundity. This result would oppose the claim made in a prior study,

which found that rats' female reproductive function and fecundity were both increased when they did eat moringa leaves [62].

The other reproductive parameters, such as the gestation index, abortifacient effect, pre-coital time (the number of days waited to be inseminated starting from pairing of the animals), number of implantations and post-implantation loss, however, were significantly affected in F0 and F1 females treated at higher doses. The changes in these reproductive parameters were consistent across the generations. The reason why these negative effects might be due to the existence of alkaloids in the plant that are dangerous and have hostile consequence on the pregnancy and used to induce abortion by physicians solely or in combination through other oxytocic agents [113]. Correspondingly, studies shows that the phenolic compounds, phytosteroids, and saponins which are active ingredients of *M. stenopetala* have abortifacient effect and antifertility potential in animal models [114]. Generally, consistent results this study indicated that *M. stenopetala* leaves extract may have potential reproductive toxicity when administered at high doses.

In addition, the pregnancy outcomes in both generations of female rats were affected following exposure to the plant extracts. In both F0 and F1 generations of animals, the number of pups delivered alive was decreased significantly in 1000 mg/kg treated groups. This finding is consistent with another previous study reported a significant decrease of the implantation sites in pregnant rats treated a higher dose of extracts from seeds, flowers, and stem of moringa plant [62]. This decrease in the implantation sites might be due to the presence of alkaloid in the plant that might cross the placental membrane and interrupt microtubule formation that plays a critical role in cell division and then affect embryogenesis [142].

In the present study, the viability index at PND4 was significantly decreased in both F1 and F2 litters treated with the higher and middle dosages. Otherwise, significant differences were not observed at PND 0 and 21. The pups were measured at PND 0, 4, 7, 14 and 21 and they did not reveal significant variations between the treated and control groups of both generations. In rodent animals, including rats, measuring the AGD is an important and critical landmark used to evaluate their developmental status. It in common is an excellent non-invasive indicator for the functional difference of the external genital organs and it is mostly used as a hormonally sensitive parameter of the close sexual differentiation [151, 162, 163]. In the current study, no significant difference was

observed in the AGD of both F1 and F2 male and female pups at PND0 and PND4. These indicated that extract of the plant may not exerted noxious consequences on the viability, weights and AGD of the pups of both generations.

Furthermore, in both F1 and F2 male and female pups, significant differences were not seen on the ages at which the pinna separated, incisor erupted, hair sprouted, and ear and eye opened between the treated and control groups. Furthermore, the durations at which preputial separation in male pups and vaginal opening in female pups were not statistically significant among the treated and control groups. Preputial separation, which is the prepuce's separation from the glans penis and folding below the glans, is used to determine whether a male rat has reached puberty [164]. These developmental milestones serve as early indications of reproductive maturity [137, 151]. From these findings, it could be possible to conclude that *M. stenopetala* leaf extract has little to no effect on the physical and functional development of F1 or F2 offspring or their sexual maturity.

In females, ovarian and extraovarian hormones are responsible for the occurrence of ovulation and maturation of the ovarian follicles. As a result, changes in the levels of these hormones could lead to the alterations in these hormones lead to indiscretion of the ovarian function and finally could affect the estrous cycle [165, 166]. For example, the cooperative functions of LH and FSH accelerate the maturation of ovarian follicles and estrogen secretion [151].

Furthermore, androgen in male plays a central role for fetal growth at a critical period for proper masculinization in both internal and external reproductive structures. Therefore, concession to the androgen because of the exposure to estrogenic agents during development might result in abnormal development of the external genitalia [163, 167]. According to the report of McLachlan et al., [168], spermatogenesis in the seminiferous tubules is stimulated by the testosterone, and FSH. Testosterone is also responsible for maturation of sperm cells in the epididymis. From this explanation, we understand that testosterone and FSH are responsible to stimulate growth and secretory functions of the male reproductive organs; therefore, the increased in the sperm count and motility in this study could be due to the significant increase of these sex hormones in the tread animals [62].

In both F0 and F1 male parental animals treated with the high dose of the plant extract, the FSH, LH and testosterone levels were significantly increased. The main reason for the increment of these hormone levels might be due to the presence of phenolic compounds, saponins, and flavonoids in the plant that have androgenic and estrogenic potential in animal models and could elevate the levels of testosterone, LH and FSH in the serum of the animals. This finding is agreed with the study reported Yousef [169] that stated the same reason. In the same way, presence of saponins in the plant might have a potential to boost the testosterone levels [62]. Therefore, the increment of these sex hormones consequently leads to increased number of sperm cells, increased their motility, and decrease the number of sperm cells with abnormal morphology.

It is known that LH is an important and a key hormone for the production of the steroids from the gonads. Therefore, the increase of LH concentration in the blood, cause increase of testestrone production in the Leydig cells [62, 170, 171]. Hence, the increased level of testestrone in the current study might be due to the stimulating effect of the plant to the Leydig cells by increasing LH production in the pituitary gland.

Differences were found in the levels of reproductive hormones, in both F0 and F1 female rats treated with the plant extract, yet it was not statistically significant. In the high dose treated groups of F0 and F1 animals, the serum FSH, LH, progesterone and estradiol levels were modestly increased.

Follicle stimulating hormone is a key hormone in mammalian reproduction that is necessary for gamete formation during the fertile stage of life as well as gonadal growth and maturation at precocious puberty. This key hormone drives the ongoing development and maturation of ovarian follicles by binding to receptors on granulosa cells. As a result, a large decrease in FSH levels suppresses follicle development and delays the maturity of follicles during the preovulatory phase [172, 173]. The possible reason for the elevated levels of FSH even though not significant, might be because of the plant extract that may exert its effect on the anterior pituitary gland that secrets FSH or hypothalamus that regulates production of FSH by stimulating gonadotropes in the pituitary gland [172, 173]. From the above rationale, it may be possible to conclude that the slightly increased FSH levels in the animals given 1000 mg/kg of *M. stenopetala* leaf extract in this study may imply that the plant has potential effects for folliculogenesis and maturation in the ovarian cortex.

The release of sex hormones from the gonads is stimulated by luteinizing hormone. Ovulation of mature follicles occurs in the ovary in females that are fertile. This ovulation occurs when a surge in LH secretion is released during the preovulatory periods. According to several studies [165, 174, 175], proestrus-stage LH release spikes are what trigger ovulation. In light of this, even though not significant, the rise in the serum LH levels in both F0 and F1 female parents may be because of the stimulating effect that brought on by the administration of the plant extract on the release of LH, which may essentially improve ovulation and ultimately the estrous cycle, conception, and reproduction in animals [62, 170, 171].

According to several investigations, normal testicular histology and sperm count are reliable indicators of fertility [176, 177]. In male animals, the gonadotropins, FSH, and LH hormones controlled the viability of sperm cells, their number, as well as their motility. In particular, the FSH increases spermatogenesis by binding to the receptors found on the Sertoli cells on the wall of seminiferous tubules [62]. This finding is in line with the study conducted by Akunna et al. [178] on the leaves of *M. oleifera*, and reported a considerable potential that increase the plasma testosterone FSH, and LH levels.

In the two-generation reproductive toxicity study, the plant extract was given for the animals for 10 weeks of premating period in both F0 and F1 parental animals, particularly in males that covers one full course of spermatogenesis period in rats. The high-dose treatment groups showed higher levels of the aforementioned hormones. Both F0 and F1 parent rat groups that had received large doses of the treatment showed tendency of increase in the number of sperm cells. The rationale behind this might be because the plant extract may promote the release of reproductive hormones in both F0 and F1 rats, which would explain the likely increase in levels of these hormones.

Furthermore, the percentage of abnormal sperm cells was reduced in 1000 mg/kg treated animals. As it is reported by different scholars, oxidative stress that could be produced during metabolism, can leads to lipid peroxidation, production of ROS, fragmentation of the genetic materials (DNA), and apoptosis that may cause abnormal sperm cells to increase [179]. The likely explanation for this observation also might be because of the flavonoids, that have antioxidant properties, and reduce oxidative stress, which lowers the number of abnormal sperm cells [62, 180]. On the basis of these results, we hypothesized

that *M. stenopetala* leaf could be used to manage reproductive problems, mostly issues involving reproductive hormones.

In the present study, changes were not observed in the absolute and relative organ weights among the treated and control groups of both F0 and F1 male and female parental animals. This might be due the presence of flavonoids in the plant that have antioxidant property that is used to prevent oxidative stress and then tissue damage in the exposed animals [62]. These suggested that ethanol extract of the plant extract might be non-toxic to male and female reproductive organs in rats.

In reality, the principal reproductive organs are highly delicate and they are subtle to toxicant induced damage. During drug toxicity evaluation, it is an important issue to assess the functional and structural integrity of the reproductive system of both sexes that might leads to problems in the gametogenesis [181]. In this study, histopathological investigations done on the male reproductive organs such as the testes, epididymis, seminal vesicles, and prostate gland and the ovary, uterus and vagina did not show any discernible differences in both F0 and F1 parental rats of the treated and control groups. This normal histopathologic feature of the reproductive organs might be due to the flavonoids present in the leaves of the plant of interest. As it is reported before, the flavonoids which is a well-known antioxidant that might prevent oxidative stress and further prevent tissue injuries and damage of organs system [62]. In addition, it is reported that moringa leaves contain abundant antioxidants such as catalase, glutathione, peroxidase, vitamin A, C, E that can delay or hinder the oxidation of other molecules and prevent cells from damage that brought on due to exposure to free radicals, mainly ROS. In addition, the presence of phenolic compounds in the plant, could scavenge free radicals and used to prevent tissue injury [182, 183]. Out of the 200 female rats studied from the F0 and F1 parents, one F0 female rat given the higher dose of the extract showed hypertrophied vaginal epithelium. It is impossible to conclude whether or not the hypertrophied vaginal epithelium is a result of the therapy since this was only seen in one rat.

In the current study, the high- and middle-dose-treated F0 and F1 parental rats showed significantly higher in the serum levels of ALT, ALP, and AST than in the pair-fed control groups. Similarly, the high-dose-treated rats had higher levels of creatinine and a significantly lower concentration of urea. Significant alterations in the results of kidney and liver function tests indicated the occurrence of chronic kidney and liver injury, even

though we did not looked at the changes in the histopathology of the liver and kidneys [184]. Accordingly, these changes in the levels of kidney and liver biomarkers might indicate chronic injuries to the liver and kidney tissues brought on due to the prolonged exposure of the animals to the plant extracts, that is suggestive to toxicity of the plant to these vital organs [185].

In F0 and F1 parental animals, treated with the high dose of the plant extract, a significant decrease of the serum glucose level was observed. This result is agreed with a previous study that reported an antihyperglycemic effects from experimental animals treated with extracts of *M. stenopetala* leaves [186, 187]. Furthermore, this backed with local residents' assertions that *M. stenopetala* leaves reduce blood sugar levels and are subsequently utilized to treat diabetes mellitus [188]. This would be expected given that the leaves of *M. stenopetala* contain terpenoids and flavonoids. According to studies, flavonoids act as an antihyperglycemic and antihyperlipidemic agent and modulate the glucose transporter protein to reduce blood sugar levels [189, 190]. This suggests that *M. stenopetala* may have antihyperlipidemic effects, and that these benefits may be brought on by the presence of flavonoids in the plant, as suggested by the drop in the serum LDL levels and the rise of HDL levels.

7. CONCLUSIONS

Higher doses of *M. stenopetala* leaf may be toxic to the developing rat embryos ad fetuses. The number of embryos and fetuses decreased in the high dose treated animals. In addition, embryonic ad fetal resorption increased in the high dose treated animals, indicating the toxicity of the plant. Furthermore, it delays the growth of fetuses and embryos. Retarded development of the sensory otic system, the yolk sac circulation, and somite scores in rat embryos showed the plant's teratogenic potential. It decreased the morphological scores and CRL in the rat embryos and fetuses treated with high doses of the tested plant extracts. Besides that, the plant extract changed the placental histopathology and reduced the fetal and placental weights.

Despite the fact that, the plat material didn't show significant changes on the postnatal developmental parameters or sexual maturation of rat offspring, it might not be safe to use while pregnant. Because it significantly affected pregnancy outcomes like pre-coital time, gestation index, number of implantations and live births. Also, the plant extract may have abortifacient potential and implantation loss. Therefore, limiting excess consumption of the plant materials, particularly the leaves during pregnancy is recommended. In addition, long-term consumption of the test plant might exert toxic insult on the kidneys and liver, as our study revealed a change in the results of kidney and liver function test.

The plant has the potential to boost male reproductive hormones, which would be advantageous. As shown in our study, the level of reproductive hormones such as FSH, LH, and testosterone in the serum of male animals given *M. stenopetala* leaf extract is increased. Furthermore, the plant might have antihyperglycemic and antihyperlipidemic properties.

8. LIMITATIONS and STRENGTH of the STUDY

Teratogenic profiles of *M. stenopetala* leaves was investigated on rat fetuses and embryos that were 20 and 12 days old, respectively. In addition, this study demonstrated the plant's developmental toxicity on many embryonic ad fetal developmental parameters like skeletal development as well as organ morphology. In this study, we also tried to assess the effects of the plant material on the reproductive parameters for F0 and F1 parental animals. Furthermore, it assessed postnatal developmental milestones in the F1 and F2 pups. As a result, it advances and enriches current knowledge regarding the tested plant's toxicity to development and reproduction. However, it is not without limitations. The suggested immunochemistry and electron microscopy were not investigated because of budgetary limitations and a dearth of sophisticated laboratories. Another drawback of the current study was that, while it would have been preferable, other organ systems besides the reproductive system had also been examined, it was not. Instead of only the organogenesis period, which was days six to twelve of the pregnancy period, it would have been better if the administration period for the teratogenicity study had encompassed the whole pregnancy period.

9. RECOMMENDATIONS

Considering the results of the current two-generation reproductive toxicity ad teratogenicity study, the following recommendations are generously provided for the concerned stakeholders:

1. **For concerned communities;**
   - It is advisable to limit consuming large amounts of the plant products, mainly the leaves, during the gestation period.
2. **For the governing bodies**
   - It is crucial to set regulatory standards regarding how traditional healers and the local community properly use medicinal plants.
   - Dose limits should be prepared in mutual collaboration with traditional healers, the Minister of Health and regional and federal public health institutes.
3. **For researchers**
   - Developmental toxicity studies for other key parts of *M. stenopetala* should be considered by giving the plant extract during the entire gestation period.
   - *In vitro* cytotoxicity (embryo culture) should be conducted.
   - Genotoxicity, study to identify mechanisms of the teratogenicity of the plant extracts.
   - Advanced studies like immunohistochemistry and electron microscopy should be conducted.
   - Further reproductive toxicity studies for other parts of the plant should be considered.
   - Reproductive toxicity on multigeneration of animals should be conducted
   - It is important to conduct Extended-one-generation reproductive toxicity that adds additional parameters
   - Further toxicity studies on additional body systems, such as the cardiovascular, endocrine, nervous, and immune systems should be conducted
   - Further experiments with nonrodent animals should be conducted.
   - Community based survey about prevalence of birth defects in the areas where the plant is used as a staple food and in folks medicine.

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11. ANNEXES

Annex 1: Preparation of Solutions and their composition Used in the Experiment

a) To make Bouin's solution,

- 25% formalin
- 75% picric acid and
- 5% glacial acetic acid were combined

b) Mall's solution: a concoction of

- 1% potassium hydroxide (KOH)
- 20% glycerol, and
- 79% distilled water

c) Formalized saline

- Sodium bicarbonate (5 g) in 99 ml normal saline
- 1 ml of 40% formalin

Annex 2: Tissue Processing approaches

In this study, tissues were processed in the Histopathology Laboratory of EPIHI, using an automatic tissue processor (Leica TP 1020).

a) **Fixation:**
   - The tissues were preserved in 10% formalin solution overnight.

b) **Dehydration:**
   - The preserved tissues were given a five-minute rinse under running water. Next, they went through increased alcohol concentration in the following ways:
     i. 40% alcohol for 1:30 hours
     ii. 70% alcohol for 1:30 hours
     iii. 80 % alcohol for 1:30 hours
     iv. 90% alcohol for 1:30 hours
     v. Absolute alcohol I, II and III for 1:30 hours in each

c) **Clearing:**
   - Clearing of the tissues were follow the of xylene I and II.
     - ✓ Xylene I stayed for 1:30 hours
     - ✓ Xylene II also stayed for 1:30 hours

d) **Infiltration**:
   - Infiltration of the tissues was done using three steps of paraffin wax I, II and III.
     - ✓ Paraffin wax I stayed for 90 minutes
     - ✓ Paraffin wax II stayed for 90 minutes
     - ✓ Paraffin wax III stayed for 90 minutes

e) **Embedding:**
   - The tissues were placed in an embedding cassette and filled with melted paraffin wax (58–60 oC)

f) Sectioning:

   - Was done using a Leica rotatory microtome, and serial sections of 5 µm thickness were made.
   - Ribbons of sections were floated in warm water (45 oC).

- Sections were spread on the smeared slides, and then the slides were dried on the hot plate at 50 ^{o}C for 30 minutes.

g) Deparaffinization:

- The slides were placed in Xylene I, II, and III in order to extract the embedded paraffin wax.
  - ✓ Xylene I for 5minutes
  - ✓ Xylene II for 5minutes
  - ✓ Xylene III for 5minutes

h) Rehydration:

- To replenish fluids, the slides were placed in decreasing alcohol grades.
  - ✓ Absolute alcohol I for 2minutes
  - ✓ Absolute alcohol II for 2minutes
  - ✓ For 2 minutes in each, the tissues pass through 90% alcohol, 80% alcohol, 70% alcohol, 50% alcohol and running tap water.

i) **Staining:** Tissue staining procedures:

i. Stained in Hematoxylin for 10 minutes.
ii. Bluing in running tap water for 8 -10 minutes.
iii. Stained in 1% eosin for 7-10 minutes.
iv. Washed in running tap water for 5 minutes
v. Dehydrated through
   - ✓ 70 % alcohol for 2 minutes
   - ✓ 95% alcohol for 2 minutes
   - ✓ absolute alcohol I for 2 minutes
   - ✓ absolute alcohol II for 2 minutes
vi. Cleared by
   - ✓ Xylene I for 5 minutes
   - ✓ Xylene II for 5 minutes
vii. Mounted – By DPX

Annex 3: Morphological Scoring System for measuring the developmental status of rat embryo [124].

Variables	**Scores and descriptions**						Total Score
	0	**1**	**2**	**3**	**4**	**5**	
Yolk sac circulation system	not visible or scattered island	corona of blood islands with/without anastomosis	vitelline vessel with few yolk sac vessels	full yolk sac plexus of vessels	yolk sac obliterated vitelline artery and vein well separated		
Allantois	allantois free in exoceolome	allantois fused with chorion	umbilical vessels	separate aortic origin of umbilical and vitelline vessels			
Flexion	ventrally convex	Turning	dorsally convex	dorsally convex with spiral torsion			
Heart	endocardial rudiment not visible or visible but not beating	beating "s "shaper cardiac tube	convoluted cardiac tube	bulbus cordis, atrium communes or ventriculus communes	dividing atrium communes		
Caudal neural	Neural plate or	closing but	neural fold closed at	posterior neuropore	posterior neuropore		

tube	fold	unfused neural fold/groove	level of somite4/5	formed but open	closed		
Hind brain	Neural plate	rhombomere A and B	anterior neuropore formed but open	anterior neuropore closed rhombencephalon formed	pronounced pontine flexure with transparent roof of 4th ventricle		
Midbrain	Neural plate	mesencephalic brain folds	closing of mesencephalic folds	completely fused mesencephalon	visible division b/n mesencephalon and diencephalon		
Forebrain	Neural plate	prosencephalic brain folds	completely fused prosencephalon	visible telencephalic evagination	well elevated telencephalic hemisphere		
Otic system	no sign of otic development	flattened otic primordium	otic pit	otocyst	otocyst with dorsa recess	otocyst with endolymphatic duct	
Optic system	no sign of optic develop	sulcus opticus	elongated optic primordiu	primary optic vesicle with open	indented lens plate	lens pocket or	

	ment		m	optic stalk		vesicle	
Olfactory system	No sign of olfactory development	olfactory plate	olfactory plate with rim	distinct olfactory ridge	lateral nasal process and medial rim		
Branchial bars	none visible	I visible	I and II visible	I, II and II visible	II overgrowing and obscure III		
Maxillary process	No sign of maxillary development	Maxillary process demarcated. Visible cleft anterior to bar I	Maxillary process fused with nasal process				
Mandibular process	No sign of mandibular development from bar I	First branchial bar fused and forming mandibular process					
Fore limb	No sign of fore limb development	Distinct evagination of wolffian crest at the level of somite 9-13	Forelimb bud	Paddle shaped for limb bud	Distinct apical ridge on forelimb		

Hind limb	No sign of hind limb development	Distinct evagination of Wolffian crest at level 01 somite's 26-30	Hind limb bud	Paddle shaped for hind bud			
Somite's	0-6	7-13	14-20	22-27	28-34	35-41	

Annex 4: Bone Ossification Assessment checklist

S. No	**Bone ossification**	Complete	Partial	Absent
1	Craniofacial bone			
2	Hyoid			
3	Clavicles			
4	Scapulae			
5	Sternebrae			
6	Ribs			
7	Thoracic vertebrae			
8	Lumbar vertebrae			
9	Tail bones			
10	Humerus			
11	Ulna			
12	Radius			
14	Carpals			
15	Metacarpals			
16	Phalanges of hand			
17	Hip bones			
18	Femur			
19	Tibia			
20	Fibula			
21	Tarsals			
22	Metatarsals			
23	Phalanges of foot			

Annex 5: Measuring Reproductive Parameters [191]

S. No	Reproductive indices	Description
1	Mating/copulation index	(No. of females mated/ no. of females placed with males) x100
2	Fertility index	(No. of females pregnant/no. of females placed with males) x100
3	Fecundity index	(No. of females pregnant/no. of females mated) ×100.
3	Gestation/pregnancy index	(No. of females with live pups/no. of females pregnant) ×100
4	Live birth index	(No. of live offspring/ Number of offspring delivered) × 100
5	Sex ratio	No. of male offspring /Number of female offspring
6	4-day survival/Viability index	(No. of live offspring at lactation day 4/ Number of live offspring delivered) × 100
7	Lactation/ Weaning index	(No. of live offspring at day 21/ Number of live offspring born) × 100
8	Preweaning index	(No. of live offspring born - Number of offspring weaned/ Number of live offspring born) × 100
9	Delivery index	(No. of live pups delivered / no. of implantations) ×100
10	Abortifacient index	(No. of aborted rats/no. of pregnant rats) ×100
11	Total post implantation loss	((No. of implantation sites−no. of live births)/no. of implantation sites) ×100.

Annex 6: Tabular summary for assessing developmental land marks [192].

Parameters	**Dosing of EtOH extract**			**Control groups**	
	250mg/kg	500mg/kg	1000mg/kg	Pair-fed control	*Ad libitum* control
Mean number of live pups/dams at birth					
Mean number of live pups/dams at day 4					
Mean number of live pups/dams at day 21					
Sex ratio (m/ f)					
Litter weight at PND 0, 4, 7, 14 & 21					
Days of vaginal opening					
Days of preputial separation					
AGD at PND 0 and 4					
Pup weight at the time of AGD measurement					
Days of incisor eruption					
Days of pinnal detachment					
Days of hair sprouting					
Days of ear opening					
Days of eye opening					